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Protein expression profiling of mouse hippocampus area CA1 and CA3 during memory formation

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Summary

The hippocampal formation is a brain structure essential for higher-order cognitive functions. It has a characteristic anatomical organization and cellular composition, and its sub-regions have different properties and functional roles. Areas CA1 and CA3 in particular, are key sub-regions for learning and memory formation that fulfill complementary but specific functions. The molecular basis for such specific properties and the link to learning and memory remain unknown. The work presented in this thesis analyzes protein expression differences between hippocampus area CA1 and area CA3 under basal conditions and changes in protein expression induced by two different learning paradigms. It provides evidence that there are extensive differences between the proteome of the two sub-regions. SWATH-MS is used to identify proteins with expression differences between area CA1 and area CA3 under basal conditions. It is further demonstrated that both learning paradigms induce changes in protein expression in area CA1 and area CA3 at multiple time-points following the tasks. The application of bioinformatics tools allowed us to interpret the expression data over the whole time-course and revealed intriguing differences between the two sub-regions. It is shown that dynamic changes in area CA1 are consistent throughout both learning paradigms. In contrast, changes induced in area CA3 by the two learning paradigms differ from each other. A more detailed analysis of area CA1 reveals a group of proteins that display similar expression characteristics in both paradigms. Members of this group are components of the electron transport chain. They show a characteristic down-regulation after two hours and up-regulation after eight hours. In the discussion we explore how these electron transport chain proteins could contribute to the role of area CA1 in episodic memory formation and what additional experiments would be necessary to demonstrate this.

Zusammenfassung

Der Hippocampus ist eine Hirnstruktur welche essenziell für kognitive Funktionen höherer Ordnung ist. Er hat eine charakteristische anatomische Organisation und zelluläre Zusammensetzung und seine Unterregionen haben verschiedenen Eigenschaften und funktionelle Rollen. Die CA1 und CA3 Regionen sind wichtige Unterregionen für Lernen und Erinnerung und vollführen ergänzende aber spezifische Funktionen. Die molekulare Basis für solche spezifischen Eigenschaften und deren Bezug zu Lernen und Erinnerung sind bis anhin unerschlossen. Diese Diplomarbeit analysiert sowohl Proteinexpressionsunterschiede zwischen den Hippocampus CA1 und CA3 Regionen unter Standardbedingungen als auch Veränderungen welche in zwei unterschiedlichen Lernmodellen induziert werden. Die Arbeit liefert Beweise, dass extensive Unterschiede zwischen den Proteomen beider Regionen existieren. SWATH-MS erlaubt die Identifikation von Proteinen welche unter Standardbedingungen zwischen den Regionen unterschiedlich exprimiert sind. Weiter wird gezeigt, dass beide Lernmodelle Veränderungen in Proteinexpression in beiden Regionen nach mehreren Zeitpunkten induzieren. Bioinformatische Tools erlauben es die Expressionsdaten über den gesamten Zeitraum zu analysieren und enthüllen faszinierende Unterschiede zwischen den beiden Unterregionen. Es wird gezeigt, dass dynamische Änderungen in der CA1 Region in beiden Lernmodellen konsistent sind. Im Gegensatz dazu sind Änderungen welche von den zwei Lernmodellen in der CA3 Region induziert werden Unterschiedlich von einander. Eine detailliertere Analyse der CA1 Region enthüllt eine Gruppe von Proteinen welche ähnliche Expressionscharakteristiken in beiden Lernmodellen aufzeigen. Mitglieder dieser Gruppe sind Komponenten der Elektronentransportkette. Sie zeigen eine charakteristische herunter Regulierung nach zwei Stunden und rauf Regulierung nach acht Stunden. In der Diskussion wird argumentiert auf welche Weise Proteine der Elektronentransprotkette für episodisches Gedächtnis wichtig sein könnten und welche weiteren Experimente nötig wären um dies zu belegen.

Contents

Glossary

ACN	acetonitrile
AD	Alzheimer disease
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATP5A	ATP synthase subunit alpha, mitochondrial
BDNF	brain-derived neurotrophic factor
BH	Benjamini Hochberg
CA	cornus ammonis
cDNA	complementary DNA
CON	control
CpG	cytosine-guanine
CREB	cAMP response element-binding protein
Da	dalton
DDA	data dependent acquisition
DG	dentate gyrus
DIA	data independent acquisition
DIGE	difference in gel electrophoresis
DNA	deoxyribonucleic acid
DNAme	DNA methylation
DNMT	DNA methyltransferase
DTT	dithiothreitol
E	embryonic day
EC	entorhinal cortex
EE	environment enrichment
emPAI	exponentially modified protein abundance index
ERK	extracellular-signal regulated kinases
ETC	electron transport chain
EtOH	ethanol
FA	formic acid
FACS	fluorescence activated cell sorting
FC	fold change
FGFR	fibroblast growth factor receptors
G	gauge
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GO	gene ontology
GTP	guanosine triphosphate
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDM	histone demethylase
HILIC	hydrophobic interaction chromatography
HPA	hypothalamic-pituitary-adrenal
HPLC	high-pressure liquid chromatography
HPTM	histone post-translational modification
IAA	iodoacetamide
IGF2	insulin-like growth factor 2
iRT	indexed retention time
iTRAQ	isobaric Tags for Relative and Absolute Quantitation
IVF	in-vitro fertilization
LC	liquid chromatography
LTP	long-term potentiation
m/z	mass to charge ratio
MALDI	matrix assisted laser desorption/ionization
MAPK	mitogen-activated protein kinase
MDD	major depressive disorder
miRNA	micro RNA
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein
MS	mass spectrometry
MS/MS	tandem mass spectrometry

MS1	first stage of tandem-mass spectrometer
MS2	second stage of tandem-ass spectrometer
mtDNA	mitochondrial DNA
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
ncRNA	non-coding RNA
NMDAR	N-methyl-D-aspartate receptor
NR1	NMDAR subunit 1
NRE	no re-exposure
Nrf1/2	nuclear respiratory factor 1/2
OLR	object location recognition
OR	object recognition
PBS	phosphate buffered saline
PGC	primordial germ cell
PGC-1α	proliferator-activated receptor gamma coactivator 1-alpha
PKA	protein kinase A
PMSF	phenylmethane sulfonyl fluoride
PND	post-natal day
ppm	parts per million
PTM	post-translational modification
RIPA	Radioimmunoprecipitation assay
RNA	ribonucleic acid
ROS	radical oxygen species
rpm	rotations per minute
RT	retention time
RT-qPCR	real-time quantitative polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of mean
sncRNA	small non-coding RNA
SRM	selected reaction monitoring
STEM	short time expression miner
Tbp	TATA-binding protein
TBS	TRIS buffered saline
TEAB	triethylammonium bicarbonate
TFA	trifluoro acetic acid
Tfam	mitochondrial transcription factor A
TOF	time-of-flight
TRIS	tris(hydroxymethyl) aminomethane
tRNA	transfer-RNA
UA	urea
UPS	ubiquitin-proteasome system
UV/V	ultraviolet-visual
VTA	ventral tegmental area
WB	western blot
Ybx1	Y-box binding p

1 Introduction

1.1 Memory

The ability to acquire information, store it and retrieve it at a later time is crucial for all higher-order life forms. It allows them to adapt to new environments and situations quickly and increases their chance of survival. The process by which information is encoded, stored and retrieved is called memory.

Memory can be further sub-divided by the time frame in which it is able to perform encoding, storage and retrieval. Short-term memory allows for fast encoding and retrieval within seconds but with limited capacity and limited storage duration. Long-term memory on the other hand allows storage of almost unlimited amount of information over long time-periods at the cost of much slower encoding. The cellular processes that mediate short-term and long-term memory are distinctively different. The molecular basis of short-term memory is not yet fully understood. A proposed model is that calcium-mediated synaptic facilitation in the recurrent connections of neocortical networks sustains short-term memory (Mongillo, Barak et al. 2008). In contrast, long-term memory depends on alteration of synaptic connections between neurons; a process that itself depends on the translation of new proteins (Costa-Mattioli and Sonenberg 2008).

Further, memory can be sub-divided by type. A general distinction is made by whether the recall of the memory is consciously performed or not. The first, consciously performed type of memory is referred to as declarative memory, while the later, the unconsciously performed, is referred to as procedural memory. Declarative memory includes further distinct types of memory, such as episodic memory, a type of memory that is specific to contexts and mediates the way past occurrences are remembered in a autobiographical way, and semantic memory, the recollection of abstract knowledge and facts. Procedural memory on the other

hand is based on implicit learning, learning without awareness of what has been learned, for example the acquisition of motor skills.

Types and sub-types of memory depend on different brain regions. The medial-temporal lobe, which consists of the hippocampus and adjacent cortical regions, largely mediates episodic memory (Eldridge, Knowlton et al. 2000, Dickerson and Eichenbaum 2010), semantic memory additionally depends on other cortical regions (Binder and Desai 2011) and the basal ganglia and cerebellum mediate procedural memory.

To sum up, memory is the ability to acquire, encode, store and recall information. Molecular mechanisms and implicated brain regions differ between short- and long-term memory and between different types of memory.

1.1.1 Episodic memory

Episodic memory is a declarative memory that builds the basis of what we generally define as 'remembering'. There are nine properties, which are used to define the exact concept of episodic memory in humans (Conway 2009). Most importantly, episodic memory contains summary records of experiences, be they sensory, conceptual, perceptual or affective. They have a perspective, usually the perspective of the observer, and represent the temporal order of occurrences within a short time-slice of experience. When accessed, they can be recollectively experienced and make autobiographical remembering specific. While episodic memory is very specific for past occurrences, most episodic memory rapidly becomes inaccessible and is eventually lost.

There are two processes through which episodic memory can be maintained over longer time-periods. The first process is known as memory consolidation, through which episodic memory is reinforced and eventually transformed into a semantic form. The second process is known as memory re-consolidation, through which previously consolidated memory becomes labile again and can be further reinforced, weakened or even modified.

Memory consolidation is further sub-divided by two distinct underlying processes. Synaptic consolidation, the process in which individual synaptic connections between neurons are selectively reinforced through molecular and cellular processes (Gal-Ben-Ari, Kenney et al. 2012) and which takes minutes to hours,

and systems consolidation, in which neuronal networks that represent a specific memory are re-arranged over time, a process that can take decades (Winocur and Moscovitch 2011).

There is still debate about the exact nature of systems consolidation, but a general consensus is that during systems consolidation episodic memory that depends largely on the hippocampus over time becomes more and more independent of the hippocampus through transformation to the neo-cortex. In this process memory is gradually brought into a more semantic form. While the standard consolidation theory argues that during this process the initial episodic form of memory is lost (Squire and Alvarez 1995), the transformation hypothesis argues that both episodic and semantic memory can co-exist (Winocur and Moscovitch 2011), whilst contextual detail remain hippocampus dependent and schematized semantic details are instated in the neo-cortex.

Memory re-consolidation is often regarded as a separate process to memory consolidation, even though the theory that they have the same underlying process is still being proposed (Nader and Hardt 2009). Proof that they should be studied as separate processes has been provided by the observation that they rely on the recruitment of independent molecular components, where hippocampus dependent consolidation depends on brain-derived neurotrophic factor (BDNF) but not on Zif268, and hippocampus dependent re-consolidation depends on Zif268 but not on BDNF (Lee, Everitt et al. 2004).

During re-consolidation recall of previously consolidated memory brings it back into a labile state (Przybyslawski and Sara 1997). In this state, the memory can be reinforced, weakened or modified, depending on the circumstances. A classical example is the ‘misinformation effect’ where witnesses to a crime or accident are provided with additional information while recalling the situation (Lacy and Stark 2013). This can strengthen the memory, in case of positive reinforcement where the additional information supports it, weaken it in cases where the information contradicts it, or even modify it and add new incorrect details that were not part of the initial memory.

To recapitulate, episodic memory is the underlying process of what we understand as ‘remembering’. It is very specific and allows recollective experiencing. Memory consolidation and re-consolidation can reinforce episodic memory or modify it,

preventing its rapid loss and transforming aspects of it into a long-term stable, schematized form of semantic memory.

1.1.2 Episodic-like memory in animal models

Even though it is assumed that primates and rodents do have similar episodic memory as observed in humans, it is impossible to gain a read-out of important aspects, such as perspective and recollective experience. To prevent confusion the term episodic-like memory is used in animal models (Crystal 2010). The difficulty of assessing episodic-like memory in animal models increases with the difference to humans. While direct interaction with non-human primates allows to study many aspects of episodic-like memory (Schwartz and Evans 2001, Beran, Menzel et al. 2016), including conscious recall (Menzel 1999), studying episodic-like memory in rodents is more restrictive.

Aspects that can be tested in rodents are ‘what, where and when’ (Dere, Kart-Teke et al. 2006), with ‘what’ being memory for details in experiences, ‘where’ being memory for spatial locations and ‘when’ being memory for temporal occurrence of experiences.

There are multiple tasks that can be used to assess these aspects of memory in rodents. For the ‘what’ recognition tasks are used, where animals have to identify previously encountered cues or associations of cues, for example objects or odors (Bunsey and Eichenbaum 1996), for the ‘where’ tasks are used where animals have to identify spatial locations in which cues or goals were encountered, and for the ‘when’ tasks are used where animals have to recognize in what temporal order cues were encountered (Fortin, Agster et al. 2002). In order to motivate the animals to reliably perform these tasks either an external incentive, such as a food reward, or intrinsic incentives, such as the natural exploration drive, are used. Concordantly to the importance of the hippocampus for episodic memory in humans, it is also the key region for episodic-like memory in animals.

To summarize, the counterpart to episodic memory in humans is referred to as episodic-like memory in animal models, some aspects of which can be tested with behavioral tasks. The hippocampus is crucial for episodic-like memory in animals.

1.1.3 Object memory and object location memory

Two forms of episodic-like memory that can be assessed with behavioral tests in rodents are memory of objects and memory of object locations. The corresponding tasks, object recognition (OR) and object location recognition (OLR), consist of two phases, a familiarization phase and a testing phase (Leger, Quiedeville et al. 2013, Vogel-Ciernia and Wood 2014). In the familiarization phase animals explore a set of objects, while in the testing phase their memory for the object or object location is tested. This is done by either exchanging a familiar object from the first phase with a novel object or by spatially displacing one of the familiar objects. In essence both tasks are one-trial tasks that don't involve repeated learning. The animals' ability to remember the initial set of objects and their locations can be assessed by observing the exploration of objects during the testing phase. It has been shown that animals spend more time exploring novel cues without external motivation (Berlyne 1950) and thus animals that display increased exploration of the novel object or the displaced object demonstrate memory for the initial setting. As for episodic memory in general, OR and OLR depend on hippocampal function.

1.2 The Hippocampus

The hippocampus is a brain region that has always played an important role in neuroscience. One of the main reasons is its distinct structure, making it distinguishable from the surrounding tissue even by eye without the aid of sophisticated instruments. Ancient anatomists referred to it as cornus ammonis, the horn of a ram. The name hippocampus was termed later in the 16th century by a Bolognese anatomist, due to its resemblance of the sea horse.

1.2.1 The structure and composition of the hippocampus

1.2.1.1 The anatomy of the hippocampal formation

Anatomically, the hippocampal formation is a well-organized structure that comprises distinct areas including the dentate gyrus (DG), the cornus ammonis (CA) CA1, CA2, CA3 and CA4 (also referred to as hippocampus proper), the entorhinal cortex (EC) and the subiculum (Anderse, Morris et al. 2006). The anatomical structure can be conceptualized as a folded sheet with an S-shaped

cross section. The EC connects to the medial temporal lobe on the distal side of the transverse axis. The subiculum is adjacent to the EC and folds under it. Adjacent to the subiculum is the CA (from CA1 to CA4), which in turn is adjacent to the DG on the proximal side. CA3, CA4 and DG fold one more time towards the opposite direction of the first fold and are situated under the CA1 and Subiculum (Figure 1-1). In the literature the terminology hippocampus is used to describe the sub-structure of the hippocampal formation that comprises areas CA1, CA2, CA3, CA4 and the DG. While CA2 and CA4 are well-defined structures that have specific functions they are much smaller than CA1 and CA3, so most studies that explore hippocampal function focus on area CA1 and CA3 and the DG.

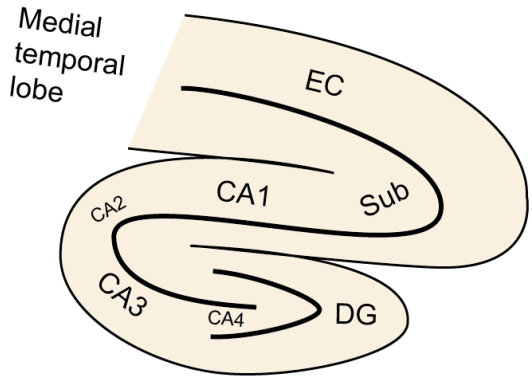

Figure 1-1: The structure of the hippocampal formation and its sub-regions

1.2.1.2 Excitatory connectivity within the hippocampus

The hippocampus receives a variety of input through the EC, which are then processed by two distinct excitatory circuits (Figure 1-2). The trisynaptic circuit (Andersen, Blackstad et al. 1966) projects information from EC layer II to both the DG and area CA3 via the preforant path. DG then projects to area CA3 through mossy fibers. Within area CA3 information flows through a recurrent network, where area CA3 neurons are connected to other area CA3 neurons through recurrent collaterals. From area CA3 information is then projected to area CA1 through Schaffer collaterals and from there to the subiculum which itself mediates the output of the trisynaptic loop. In parallel there is the direct pathway, in which monosynaptic connections project from EC layer III to area CA1 (Witter, Groenewegen et al. 1989). From there projections to the subiculum and back to EC layer V/VI mediate the output of the pathways.

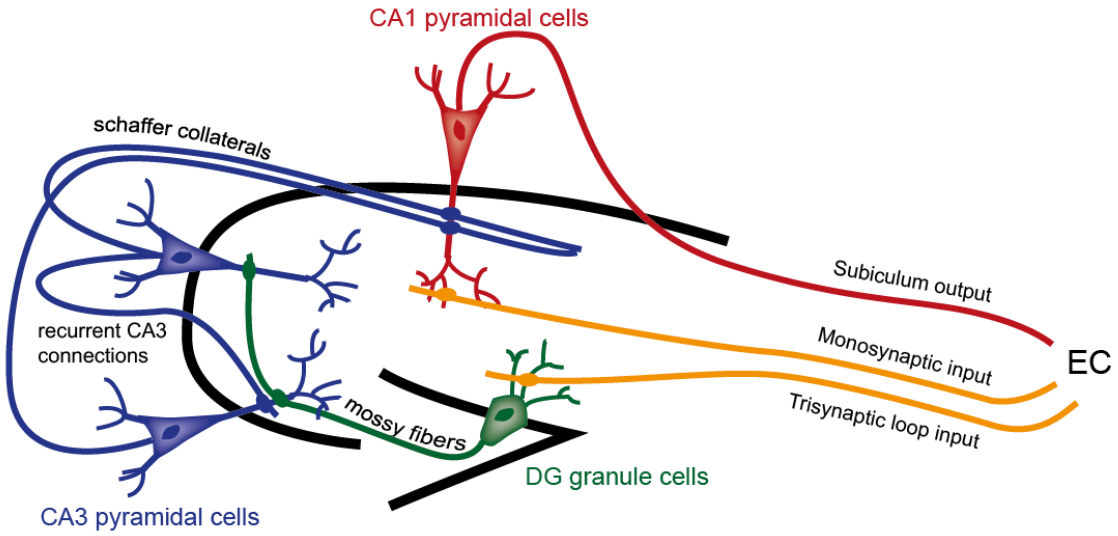

Figure 1-2: Excitatory connectivity within the hippocampus. The hippocampus receives excitatory inputs from the EC that innervate two distinct pathways, the trisynpatic circuit and the monosynaptic preforant CA1 path. Projections to the subiculum and back to EC mediate the output of the pathways.

1.2.1.3 Cell types within the hippocampus

Not only are hippocampal sub-regions distinguishable by anatomy and connectivity, they also harbor different types of cells. While CA and DG both contain glutamatergic excitatory neurons (~90% of the total neuron population) as well as GABAergic inhibitory interneurons (~10% of the total neuron population), the most pronounced differences can be found on the size and shape of excitatory neurons. Excitatory neurons mediate hippocampal information processing in both the trisynaptic circuit and the monosynaptic EC-CA1 connections. The excitatory neurons of the DG are granule cells, which are tightly packed in the granule cell layer, have a relatively small elliptical cell body and a cone-shaped dendritic tree. In contrast CA regions contain pyramidal excitatory neurons. They are larger in size compared to granule cells, have a pyramid-shaped soma and large, apical dendrites. Even though all CA areas have the same type of excitatory neurons, there are differences between area CA1 and area CA3 pyramidal neurons. While there are the obvious differences in connectivity - CA3 pyramidal neurons can form recurrent connections with other CA3 pyramidal neurons, a property CA1 pyramidal neurons lack – there are additional differences in morphology, such as

maximal branch order, remote bifurcation angle, topological asymmetry, soma surface and more (Scorcioni, Lazarewicz et al. 2004).

Even more heterogeneous is the population of interneurons in all hippocampal sub-regions. At least 16 morphologically distinct interneurons can be identified in area CA1 alone (Parra, Gulyas et al. 1998) with a similar number being proposed in the DG and area CA3. Within the whole hippocampus interneurons receive input from both excitatory as well as inhibitory hippocampal neurons (both ipsi- and contralateral) as well as from external afferents. Hippocampal interneurons project mainly locally to other cells within the hippocampus and are thought to be important for the synchronization of hippocampal excitatory neurons (Jones and Yakel 1999).

While excitatory and inhibitory neurons mediate the main function of the hippocampus they only make up roughly half of the total volume. The other half is made up by glial cells, which encompass astrocytes, oligodendrocytes and microglia. Even though they are on average smaller in size than neurons, with a glial to neuron ratio of 2:1 they are more numerous in the mouse hippocampus (Oliveira-da-Silva, Vieira et al. 2009). This ratio is more or less consistent throughout all hippocampal sub-regions and fluctuates from ~2.1 in area CA3 to ~1.9 in area CA1. For a long time the function of glial cells was assumed to be homeostatic support, myelination and protection of neurons, but recent studies have demonstrated that astrocytes contribute to both synaptic transmission and synaptic plasticity in the hippocampus (Ota, Zanetti et al. 2013).

To recapitulate, the cellular components of the hippocampus can be subdivided into three rough groups, excitatory neurons, inhibitory neurons and glial cells. Of these the most striking differences between the sub-regions can be observed for excitatory neurons.

1.2.1.4 Projections from other brain regions to the hippocampus and vice versa

The hippocampus receives input from multiple brain regions. Glutamatergic excitatory input arrives from the neocortex first projecting to the parahippocampal and the perirhinal cortex and from there to the EC (van Groen, Miettinen et al. 2003). Additionally, both cholinergic and GABAergic fibers from the septum project

to the hippocampus directly (Khakpai, Nasehi et al. 2013). Serotonergic input arrives directly form the raphe nucleus (McKenna and Vertes 2001), norepinephrine and dopamine projections arrive from the locus coreuleus (Kempadoo, Mosharov et al. 2016) (Lipski and Grace 2013) and additional dopaminergic projections arrive from the retrorubral field, the substantia nigra and the ventral tegmental area (VTA) (Gasbarri, Sulli et al. 1997).

While the hippocampal formation receives input from multiple brain regions, its output innervates both the neocortex and parts of the limbic system. Output to the cortex and the amygdala originates at the EC. Other parts of the limbic system, such as the hypothalamus and the septum, as well as the nucleus accumbens receive hippocampal projections from the subiculum through the fornix (Kelley and Domesick 1982).

1.2.1.5 The dorsal-ventral axis of the hippocampus

Even though the basic intrinsic circuit is maintained throughout the hippocampus along the dorsal-ventral axis (Figure 1-3), there are pronounced differences between the dorsal part and the ventral part. These differences can be sub-divided into three general groups. First of all, there are pronounced differences in connectivity with hippocampal projection areas. This holds true for projections to the amygdala, the nucleus accumbens and the lateral septum. The dorsal hippocampus projects predominantly to the lateral amygdala, the medial parts of the nucleus accumbens and the dorsal part of the septum, while the ventral hippocampus projects predominantly to the medial amygdala, the lateral parts of the nucleus accumbens and the ventral part of the septum (Strange, Witter et al. 2014). Furthermore, there are differences between the functions of the dorsal and ventral hippocampus. While the dorsal part has been implicated in memory, spatial perception and navigation (Moser, Moser et al. 1993, Moser, Moser et al. 1995), the ventral part is important for anxiety-related behavior (Bannerman, Grubb et al. 2003). Then, there are differences on the molecular level. The expression of many genes varies between dorsal and ventral parts of the hippocampus (Thompson, Pathak et al. 2008). The list of genes with expression differences includes axon guidance and cell adhesion molecules, ion channels and transcriptional regulators. While classically the dorsal and the ventral hippocampus are often studied

independently, it has to be mentioned that the differences between them follow a gradient along the dorsal-ventral axis of the hippocampus. There is no distinct point where the dorsal hippocampus becomes the ventral hippocampus and vice versa, there is a gradual transition between the two.

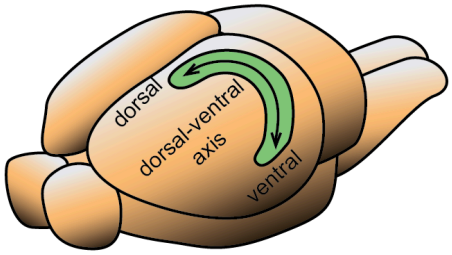

Figure 1-3: The dorsal-ventral axis of the hippocampus. The Figure depicts a mouse brain with the left hemisphere of the mouse hippocampus shown in green. The dorsal-ventral axis runs along the whole length of the hemisphere as indicated by the arrow

1.2.2 The function of the hippocampus

Over the past decades multiple theories about the function of the hippocampus have been proposed. These include a potential role of the hippocampus in behavioral inhibition, the role of the hippocampus in anxiety and stress, the role of the hippocampus in the neural representation of space and the role of the hippocampus in memory.

1.2.2.1 The hippocampus and behavioral inhibition

McNaughton and Gray (Gray and McNaughton 1982) have proposed the theory that the hippocampus plays an important role in behavioral inhibition over 30 years ago. A key argument for the theory was the fact that classical anxiolytic drugs, such as benzodiazepines and barbiturates, induce similar behavioral phenotypes in animals that can also be observed following hippocampal lesions. Additionally, these drugs were also shown to alter the theta activity within the hippocampus. The current theory, as proposed by McNaughton and Gray, is that the function of the hippocampus is to resolve conflicts between highly attractive concurrent goals and reduce the effects of interference between these goals. More specifically, they propose that the hippocampus has one general function, which is to select which behavior is the most urgent and focusing on it by suppressing all other behaviors. They argue that all other observations and theories of hippocampal function could be explained by this characteristic. Due to the theories generalizing proposition

and oversimplification of hippocampal function it received a lot of critical opposition.

1.2.2.2 The role of the hippocampus in the neural representation of space

Single cell recording of excitatory neurons in the dorsal hippocampus of freely moving rats lead to the discovery of so-called place cells (O'Keefe 1976). These neurons are active whenever the animal is in a certain location, which is termed the place field of the respective neuron. Place fields are very specific to an environment, and sudden alterations to the environment can distort, rotate or abolish place fields (Moser, Kropff et al. 2008). Especially distal cues and geometric boundaries have a strong impact on place fields. On the other hand, smooth transformations from one environment to another will often preserve place fields (Leutgeb, Leutgeb et al. 2005) demonstrating that they are somewhat plastic. With the discovery of place cells, it was proposed that the hippocampus could act as a spatial map, allowing a neural representation of the environment and enabling navigation through it. Further evidence for the spatial map hypothesis was contributed by the discovery of grid cells, hippocampal excitatory neurons that are active whenever a freely moving animal transverses the nodes of a repetitive triangular grid (Hafting, Fyhn et al. 2005). Additionally, some hippocampal neurons, termed head direction cells, display selective activity for the direction the animal's head points to (Taube 1998). Further studies in bats freely flying in a three-dimensional environment have demonstrated the presence of cells with angular tuning of the goal direction (Sarel, Finkelstein et al. 2017), where the goal is a location in the testing room containing a reward. Together, these studies support a model, where a sub-set of excitatory hippocampal neurons represents spatial aspects of the environment, the current orientation of the animal within the environment and the direction toward a goal that the animal navigates to.

While the spatial map theory of the hippocampus is supported by strong evidence, the firing pattern of hippocampal neurons is not exclusive to spatial cues. Olfactory (Eichenbaum, Kuperstein et al. 1987) and auditory (Sakurai 1996) cues can have similar importance for place field, leading to an expanded theory where the hippocampus allows representation of concurrent cues irrespective of their type (Eichenbaum, Dudchenko et al. 1999).

1.2.2.3 The hippocampus and memory

The importance of the hippocampus for learning and memory has been proposed more than half a century ago due to a case study of patient H.M. whose hippocampal formation and other parts of the medial temporal lobe were removed as treatment for severe epilepsy (Scoville and Milner 1957). This treatment resulted in a severe case of anterograde amnesia, the inability to form new memories of events after the surgical procedure. Nevertheless, recall of older information from events prior to the removal of the hippocampus was still possible to some degree. The patient also showed no impairments in cognitive abilities, working memory and motor skill learning. Numerous studies have examined the effect of introduced hippocampal lesions in animal models on episodic-like memory. Hippocampal lesions in rats impair the association of spatial cues with other spatial or non-spatial cues (Cho and Kesner 1995, Langston and Wood 2010). While these studies claim that the hippocampus is specific for association of spatial cues with other cues, another lesion study has demonstrated similar impairments for association of non-spatial cues, in this case two odors (Bunsey and Eichenbaum 1996). Association tasks depend on two distinct functions of memory. First, the co-appearance of two cues has to be encoded. Then, in a testing session, the memory has to be retrieved to decide if the presented cues are associated.

While lesion studies demonstrate that the hippocampus is required for association learning, the question remains if it has a role in memory encoding, memory retrieval or both. A prominent mechanism of memory encoding in the hippocampus is *N*-methyl-D-aspartate receptor (NMDAR) dependent long-term potentiation (LTP) (Grunwald and Kurthen 2006). This mechanism requires the co-activation of both the post and pre-synaptic neuron of a synapse. Once co-activated, the synapse is strengthened through intracellular mechanisms. LTP provides a logical explanation for association learning, since the connections between two neurons that are activated by different cues are strengthened through co-observation of the two cues. It has been shown, that NMDAR blocking in the dorsal hippocampus during the encoding phase, but not the retrieval phase is sufficient to impair association learning (Day, Langston et al. 2003), demonstrating that the hippocampus has a function in encoding of association memory. This leaves the

second question open, if the hippocampus also contributes to memory retrieval. Indeed, a number of studies have demonstrated that the hippocampus also plays an important role in memory retrieval, which depends on other molecular regulators than memory encoding (Eldridge, Knowlton et al. 2000, Vanelzakker, Zoladz et al. 2011).

Not only the learning of associations, but also the learning of the correct order of spatial locations is impaired in rats with hippocampal lesions (Chiba, Kesner et al. 1994). While this could be interpreted as impairment in spatial perception, experiments with sequences of non-spatial cues demonstrate the same impairments (Fortin, Agster et al. 2002), demonstrating that the rodent hippocampus also mediates temporal aspects of episodic-like memory.

To summarize, the rodent hippocampus plays an important role in spatial and non-spatial association memory, and is required for both encoding and retrieval of association memory. Additionally, the hippocampus is crucial for temporal order memory.

1.2.2.4 The hippocampal sub-regions and memory

The importance of the hippocampus for episodic-like memory poses the question of how hippocampal sub-regions contribute to this function. Since they have distinct structural differences the hypothesis that they mediated different aspects of episodic-like memory has been extensively studied. Sub-region specific lesions, by stereotaxic injection of either ibotenic acid or colcichine, have been used to assess memory impairment in tasks where components of episodic-like memory are tested, such as details of cues, location of cues or the temporal order of cues. Lesions of area CA1 had stronger impact on temporal order recognition (Gilbert, Kesner et al. 2001, Hoge and Kesner 2007, Hunsaker, Lee et al. 2008), but also impairments in odor-object associations have been reported (Kesner, Hunsaker et al. 2005). On the other hand, lesions of either DG or area CA3 lead to impairment in tasks where animals have to recognize locations of rewards or objects (Gilbert, Kesner et al. 2001, Lee, Hunsaker et al. 2005, Gilbert and Kesner 2006). These studies indicated, that the DG-CA3 network is more important in recognition of spatial cues of contexts, while area CA1 is more important for sequence and detail recognition. While lesion studies provide a good indication for general functions of

sub-regions, they have to be interpreted with caution. Induced lesions can either be incomplete or have an effect on surrounding tissue as well. Additionally, lesions cannot assess cellular or sub-cellular mechanisms that are important for different components of episodic-like memory.

Better specificity can be achieved with sub-region specific knockout of receptor genes that have been shown to be crucial for memory in the hippocampus. In gene knockouts selected genes of an organism are made inoperative which allows studying potential functions of the inoperative gene(s). Even better specificity can be achieved with conditional knockouts, where the activity of a gene can be made inoperative at a desired time-point. Mouse models with conditional *NR1* knockout in area CA1, area CA3 and DG have been established. *NR1* is a crucial subunit of the NMDAR and its knockout impairs NMDARs.

A mouse model with conditional *NR1* knockout in CA1 pyramidal cells has been used to verify that NMDARs in pyramidal CA1 neurons are necessary for temporal order recognition and memory (Huerta, Sun et al. 2000). Similar mouse models have been established with specific conditional NMDAR knockouts in CA3 pyramidal cells and DG granule cells. *NR1* knockout in area CA3 pyramidal neurons has been used to demonstrate that it is required for the association between novel locations and novel cues (Rajji, Chapman et al. 2006). When associating familiar cues with novel locations or familiar locations with novel cues, *NR1* knockout in area CA3 pyramidal neurons was not sufficient to impair the animals ability to do so. These findings indicate, that area CA3 might be crucial for the rapid encoding of multiple concurrent novel stimuli, but it has to be noted that the knockout efficiency in this study was only ~30%, which might be sufficient to impair association of two novel stimuli, while an even stronger decrease could be sufficient to impair association of familiar stimuli as well. Another study has demonstrated that NR1 knockout in area CA3 impairs associative memory recall in mice (Nakazawa, Quirk et al. 2002). In contrast to controls, mice with NR1 knockout in CA3 pyramidal neurons showed decreased CA1 place cell activity following partial cue removal of a familiar environment, leading to the hypothesis that CA3 can recall encoded context from a partial representation. This process, where partial information enables the recall of the full representation is known as pattern completion.

Conditional *NR1* knockout in DG granule cells has shown no impairment in spatial learning tasks, such as the morris water maze, but has been demonstrated to be important for the discrimination between two contexts (McHugh, Jones et al. 2007). The process that allows distinction between two similar contexts is known as pattern separation.

Additional studies have examined cellular activity within hippocampal sub-regions during behavioral tasks in rats using electrophysiology or early gene imaging. Increased activity in area CA1 pyramidal neurons has been reported during novelty encounters in freely moving rats in both object recognition tasks and object location recognition tasks using tetrode measurements (Larkin, Lykken et al. 2014). Imaging of c-Fos, an early gene activated by neuronal activity, demonstrated a correlation between c-Fos expression in area CA1 pyramidal neurons of rats and performance in object recognition and object location recognition tasks (Mendez, Arias et al. 2015). Similar findings have been reported in exploration of altered environments, where c-Fos expression in area CA1 pyramidal neurons of rats and the extent of alteration were correlated (VanElzakker, Fevurly et al. 2008).

Tetrode measurements in area CA3 pyramidal neurons have demonstrated firing behaviors that were very specific to contexts (Leutgeb, Leutgeb et al. 2004). Early gene imaging studies with c-Fos demonstrated activation of CA3 pyramidal neurons following spatial learning and impairment of spatial memory encoding when c-Fos was inhibited (He, Yamada et al. 2002).

Overall, these studies suggest a model where area CA1, area CA3 and DG mediate different aspects of episodic-like memory. Area CA1 is important for recognition of novelty and encoding of temporal order memory. Area CA3 mediates rapid encoding of novel contexts and pattern completion, whereas DG mediates pattern separation.

1.2.3 Hippocampus transcriptomics and proteomics

While a wealth of data describes the functional differences between proteomic sub-regions and their role in memory formation, the molecular underpinnings of these functions are largely unknown. Transcriptional studies have analyzed the transcriptome of are CA1 and CA3 under basal conditions (Lein, Hawrylycz et al.

2007, Newrzella, Pahlavan et al. 2007, Cembrowski, Wang et al. 2016) and recent technologies have even enabled cell specific transcriptional profiling in the hippocampus area CA1, CA3 and DG (Shah, Lubeck et al. 2016) using an in situ 3D multiplex imaging approach. This demonstrated, that even within sub-regions, pyramidal neurons are in distinctively different transcriptional states, where cells in area CA1 and DG tend to be more homogeneous, while cells in area CA3 display higher heterogeneity. While transcriptional data have shown sub-region specific differences at basal conditions, protein expression does not perfectly correlate with mRNA expression (Gygi, Rochon et al. 1999).

There is currently a lack of data describing the proteome of hippocampal sub-regions both under basal conditions and during the process of memory formation. Only one study has examined protein expression differences between rat area CA1 and area CA3 at baseline and following hypoxia using 2-D gel MALDI-MS and found 15 proteins with different expression between area CA1 and CA3 at baseline (Gozal, Gozal et al. 2002), but only a total of 100 proteins could be quantified in this study and no multiple testing corrections were applied to the statistical results leaving the question open of how extensive differences in protein expression between area CA1 and area CA3 are.

Not only are protein expression differences at basal levels between area CA1 and area CA3 expected, but there is also clear evidence that *de novo* protein synthesis is required in the hippocampus for the encoding of episodic-like memory (Morris, Inglis et al. 2006). Nevertheless, only a hand full of studies has looked at memory dependent changes in protein expression in the hippocampus. One recently published study examined protein expression changes in the whole hippocampus following a spatial memory task (radial arm maze) over multiple days (Borovok, Nesher et al. 2016) and identified proteins with altered expression during the memory acquisition phase (day 1), the steep learning improvement phase (day 3) and the final curve of the learning phase (day 5). While yielding interesting results this study analyzed protein expression following multiple consecutive learning experiences and used naïve home cage mice as control group, making it impossible to distinguish between learning induced effects and effects induced by exploration and increased activity. Additionally, the time-points cover long-term effects, but short- or medium-term effects in the order of hours following the learning task are not described. Another study has examined the temporal protein

expression profile in the rat DG during memory consolidation following morris water maze using 2D-DIGE (difference in gel electrophoresis) followed by excision of significantly altered spots and characterization with MS (Monopoli, Raghnaill et al. 2011). They demonstrated changes over a one-day period at multiple time-points (3, 6, 12 and 24 hours) that encompass both medium-term and long-term effects, with enrichments for cellular structure components and proteins involved in cellular metabolism. Animals in this study were sacrificed at their corresponding time-point with only one control group making it impossible to control for circadian effects. Another study analyzed the effect of fear conditioning on hippocampal protein expression in the synaptic membrane (Rao-Ruiz, Carney et al. 2015). They used a “delayed-shock” group for memory formation and a “immediate-shock” group as stress control and analyzed protein expression using iTRAQ after one and four hours. They report no changes in protein expression following one hour, but extensive changes after four hours.

To conclude, only a hand full of studies analyzed hippocampus or DG wide changes in protein expression following memory tasks. They all demonstrate that memory tasks are sufficient to induce protein expression changes that are strong enough to be detected using available mass-spectrometric approaches. Currently, there is no published quantitative proteomic data that describes proteome wide differences between area CA1 and CA3, both at basal levels and following memory tasks. The aim of this thesis is to use proteomic state of the art techniques to fill this gap and further the understanding of area CA1 and CA3 and their contribution to learning and memory.

1.3 Proteomics

1.3.1 Liquid chromatography tandem mass-spectrometry

Mass-spectrometry for protein identification and quantification is currently the only viable approach for proteome wide assessments. Alternative approaches, such as western blot or immune assays rely on the use of specific antibodies for target proteins, which gives them a limited scope. In the last decades the field of mass-spectrometry has seen constant improvements in measurement techniques and instrument sensitivity. Currently, most mass-spectrometric approaches rely on liquid chromatography coupled to a tandem mass-spectrometer (LC-MS/MS)

(Aebersold and Mann 2003). These approaches rely on the proteolysis of protein extracts with endoproteinases, enzymes that break the bonds of nonterminal amino acids, which are specific for certain amino acids. The resulting peptides are then separated with a liquid chromatography (LC) along a time-gradient and measured on a tandem mass-spectrometer (MS/MS). During the measurement process peptides are first ionized and then separated by their mass to charge (m/z) ratio in the first stage of the mass-spectrometer (MS1). Subsequently, selected precursors (intact peptide ions) are fragmented in the collision zone and the m/z ratios of resulting fragment ions are measured in the second stage of the mass-spectrometer (MS2). The resulting combination of elution time on the LC, precursor m/z ratio and fragment ion spectrum is often very specific for a given peptide and algorithms can be used to determine its sequence and subsequently the protein(s) it originated from. This characteristic is used in discovery driven (shotgun) proteomics to determine protein composition of complex mixtures.

While discovery driven proteomics are a powerful tool to determine proteome composition of a biological system, they are not ideal for quantitative assessments. One of the main down-sights is the ability to reliably measure MS2 spectra of a large amount of co-eluting peptides. At a given cycle, which is the time it takes for the mass-spectrometer to perform a MS1 measurement, determine which precursors should be selectively measured, and perform all subsequent MS2 measurements, only for a sub-set of all present precursors the MS2 spectra can be acquired. Since this method uses data dependent acquisition (DDA) of MS2 spectra the resulting data is stochastic and thus inadequate to calculate peak areas of fragment ions. Methods such as spectral counting (Balbuena, Demartini et al. 2014) the exponentially modified protein abundance index (emPAI) (Ishihama, Oda et al. 2005), or isobaric tagging of peptides (Ross, Huang et al. 2004) can be used to gain a quantitative readout from shotgun data, but the sensitivity of these approaches is low compared to other approaches, especially for smaller changes (Dowle, Wilson et al. 2016).

Higher sensitivity can be achieved with targeted approaches such as selected reaction monitoring (SRM) that measures selected precursor ions and fragment ions in each cycle of a pre-defined time window (Lange, Picotti et al. 2008) and thus allow for peak area calculation. While a powerful tool for reliable and sensitive quantification of selected proteins in complex mixtures this approach has several

limitations, being both time intensive in development, dependent on previously acquired data and limited in scope.

1.3.2 SWATH-MS

Since older quantitative proteomic methods either sacrifice sensitivity or scope alternative approaches have been developed to overcome both these limitations. One powerful method for data-independent acquisition (DIA) is SWATH-MS (Gillet, Navarro et al. 2012), which allows sensitive, consistent, and accurate quantification with no limitation in the number of quantified proteins, as demonstrated both in cell culture (Selevsek, Chang et al. 2015) and tissue extracts (Huang, Yang et al. 2015). In order to achieve reliable measurement of all fragment ions within a short enough cycle, precursors within a given range (usually 20-25 m/z) are fragmented together and the resulting MS2 of all fragments is acquired on a high resolution instrument. Within one cycle the instrument acquires MS2 spectra of all precursor windows within a given m/z range. Then, targeted data extraction is used to acquire the transitions (precursor-fragment pairs) from the individual runs. For this, prior information about peptide fragment spectra is needed, which is usually obtained from external spectral libraries, or preferably from spectral libraries generated from the same samples on the same instrument with a DDA method. Software for automated peak picking, such as mProphet (Reiter, Rinner et al. 2011), Skyline (MacLean, Tomazela et al. 2010) or Spectronaut are then used to identify the correct peaks, remove interferences and quantify transitions.

2 Subregion-Specific Proteomic Signature in the Hippocampus for Memory Formation in Adult Mice

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2.1 Abstract

The hippocampal formation is a brain structure essential for higher-order cognitive functions. It has exquisite differences in anatomical organization and cellular composition, and hippocampal sub-regions have different properties and functional roles. Areas CA1 and CA3 in particular, are key sub-regions for learning and memory formation that fulfill complementary but specific functions. The molecular basis for such specific properties and the link to learning and memory remain unknown. Here using a SWATH-MS proteomic approach and bioinformatic tools, we identify a selective proteomic signature in area CA1 and CA3, and reveal their specific dynamics during memory formation. We show that 30% of all quantifiable proteins are differentially expressed in area CA1 and CA3 at baseline, and that each proteome responds differently during the formation of memory for object or object location. Using clustering and cross-correlational analyses, we outline specific temporal proteomic profiles and an increased correlation between both forms of memory within area CA1, but not within area CA3. These results provide new insight into a proteomic basis for hippocampal sub-region molecular and functional specificity.

2.2 Introduction

De novo protein synthesis is required in the brain for the formation of long-lasting memory (Squire 2006). New proteins are needed to sustain synaptic and structural plasticity, and to stabilize synaptic connections for signal transmission between neurons (Yang, Pan et al. 2009). Complex organisms have elaborate molecular systems to control protein expression. They operate by activating or inactivating gene transcription and/or translation at specific time points, allowing proper development, cellular differentiation and functions in resting conditions and after stimulation (Molfese 2011, Hu, Wang et al. 2012). Identifying these features is a prerequisite for a full understanding of the intimate mechanisms of memory control**.**

The hippocampal formation is a forebrain structure essential for learning and memory formation. Lesion studies in human and animals have demonstrated that damage to the hippocampus impairs the acquisition of information and its storage in memory. It induces severe anterograde amnesia, the inability to form new long-lasting declarative memory, in human and leads to learning and memory deficits in rats, mice and monkeys (Brown, Warburton et al. 2010). Electrophysiological analyses in rodents showed that certain hippocampal neurons, so-called place cells, are specifically activated when an animal is in a given location, and encode spatial information (Moser, Kropff et al. 2008). Place cells are also recruited to process other types of information including olfactory and sensory signals (Eichenbaum 2004), making the hippocampus a key structure for the perception of various forms of sensory information and the formation of different types of memory. The hippocampal is particularly required for memory for context and space. It is needed for locating and identifying objects, and for navigating in an environment (Vann and Albasser 2011). In pathological conditions such as during social stress and depression, hippocampus-dependent memory performance involving contextual and spatial information is altered (Buwalda, Kole et al. 2005, Heckers and Konradi 2010, Goeldner, Ballard et al. 2013).

Anatomically, the hippocampal formation is a well-organized structure that comprises distinct areas including the dentate gyrus (DG), the cornus ammonis (CA) CA1, CA2 and CA3, the entorhinal cortex and the subiculum which have specific structural connectivity and functional properties (Anderse, Morris et al.

2006). Although both CA1 and CA3 areas contain excitatory glutamatergic pyramidal neurons and inhibitory GABAergic interneurons, they have marked differences in connectivity and functions. CA3 pyramidal neurons can form recurrent connections with other CA3 neurons, but CA1 pyramidal neurons cannot. CA3 neurons contribute to pattern completion, the ability to map incomplete or noisy patterns to complete a full pattern. Instead, CA1 neurons are thought to match output from area CA3 with afferent, unprocessed input from the entorhinal cortex (Guzowski, Knierim et al. 2004) and allow encoding of the temporal order of different contexts (Hoge and Kesner 2007). Although many studies have examined the difference between area CA1 and CA3 in terms of contribution to different types of memory, connectivity and molecular characteristics, the nature of their respective proteome and its response to learning experiences have not been studied. Here, we demonstrate that area CA1 and CA3 have different proteomes in the adult mouse brain, with distinct and specific dynamics during memory formation, revealing a yet unknown property of hippocampal sub-regions relevant for memory formation.

2.3 Results

2.3.1 The proteome of area CA1 and CA3 are markedly different in basal conditions

To determine the proteomic specificity of each hippocampal subregion, we first characterized the whole proteome in area CA1 and CA3 in basal conditions. Basal conditions in this case are when animals are placed in the experimental arena and habituated to the arena for the same amount of time as animals used for behavioral training and testing to allow consistency across the study (Figure 2-1A). We used a recently developed SWATH-based proteomics technology to conduct accurate and reproducible proteome quantification (Gillet, Navarro et al. 2012). SWATH-MS measurements of 6 biological replicates revealed high reproducibility with Pearson correlation coefficients between biological replicates of 0.985-0.997 at the protein level in both area CA1 and CA3 (Figures 2-S1 and 2-S2). Overall, we quantified 1994 proteins across samples, with an 85% overlap (1697 proteins) between area CA1 and CA3 (Figure 2-1B). We observed that some proteins are specific for area CA1 or CA3 and were quantifiable in only one of the two subregions. Thus, 238 proteins specific for area CA1 and 59 proteins specific for area CA3 were detected. To determine whether the quantified proteins are distinct or similar in area CA1 and CA3, a cellular component analysis of quantifiable proteins was conducted. The results showed that both proteomes have similar composition (Figure 2-1C). However, a statistical comparison by MSstats followed by Benjamini Hochberg adjustment showed that 532 proteins have a different level of expression in area CA1 and CA3 (31.3% of all proteins) (Figure 2-1D). Expression differences between area CA1 and CA3 similar to mRNA differences previously described (Newrzella, Pahlavan et al. 2007) were observed for several top hit proteins (Figure 2-S3). Proteins with higher expression in area CA1 in both studies include *Itpka*, a regulator of inositol polyphosphates that controls morphology of hippocampal dendritic spines (Koster, Leggewie et al. 2016), *Ntm,* a protein important for neurite outgrowth and adhesion (Gil, Zanazzi et al. 1998)*, Efhd2,* a negative regulator of NF-κB signaling which modulates synapse formation (Borger, Herrmann et al. 2014), and *Gap43*, a major component of growth cones associated with spine growth (Frey, Laux et al. 2000).

Proteins with higher expression in area CA3 include *Cpne4*, a calcium-dependent phospholipid-binding protein, *Synpr,* a membrane protein of small synaptic vesicles (Knaus, Marqueze-Pouey et al. 1990)*, Hpcal1,* a neuron-specific calcium-binding protein*,* and *Ncald*, another neuronal calcium-binding protein. GO analyses using a web-based gene set analysis toolkit (Zhang, Kirov et al. 2005) on all proteins with significant differences revealed increased expression of proteins related to cytoskeletal organization in area CA1, and increased expression of proteins related to mitochondrial functions in area CA3 (PDF S1). These results indicate that area CA1 and CA3 have pronounced differences in proteomic profile in basal conditions.

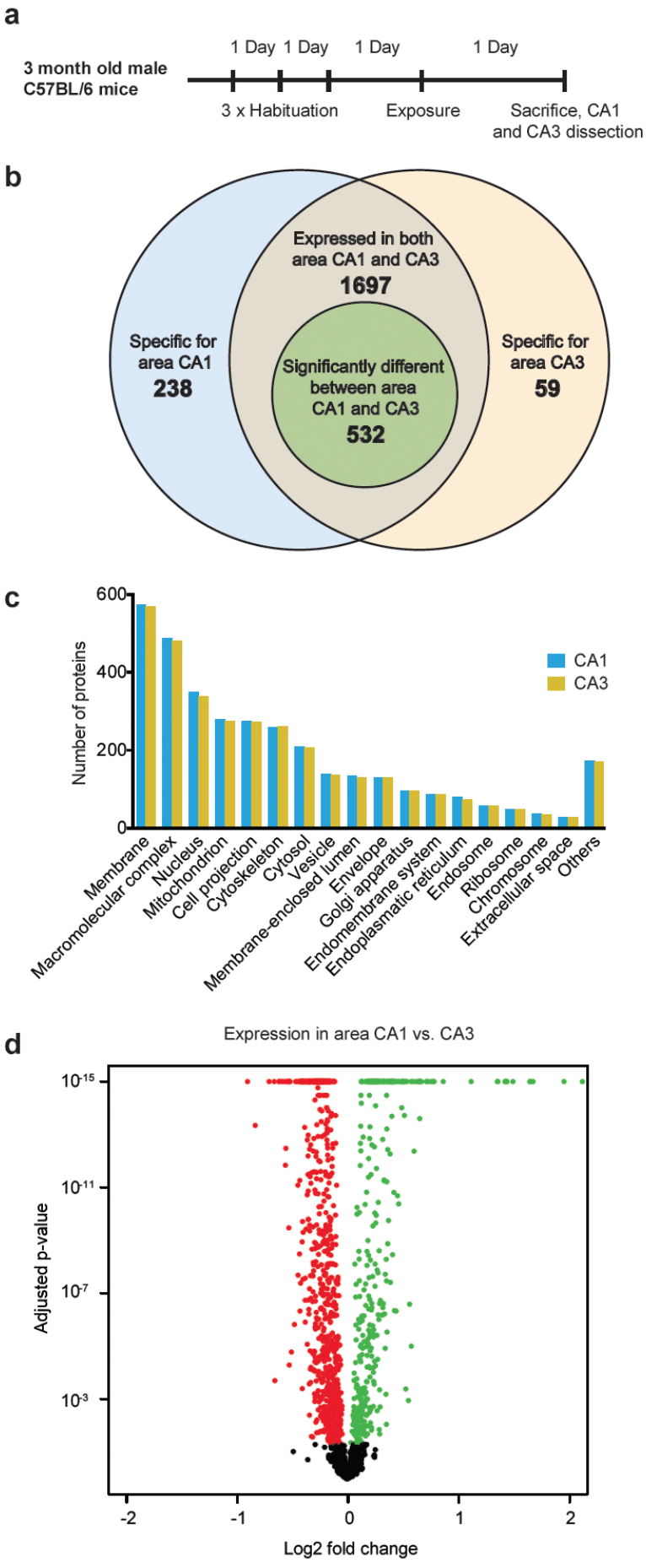


Figure 2-1: Differences between proteomes in area CA1 and CA3 at baseline.

(a) Comparison between area CA1 and CA3 proteomes in mice habituated to an arena. Animals received one habituation session per day on three consecutive days followed by an exposure session (initial exploration of three novel objects). Animals were sacrificed and area CA1 and CA3 were dissected 24 hours after exposure.

(b) Venn diagram showing overlap between the proteome of area CA1 and CA3. Proteins that are identifiable and specific for either area CA1 or CA3, identifiable and expressed in both area CA1 and CA3, or significantly expressed differentially in area CA1 and CA3 (BH adjusted p-value < 0.05, log2 FC cut-off 0.2) are shown.

(c) Cellular component analysis of proteins expressed in area CA1 and CA3 showing no difference between the composition of area CA1 and CA3 proteomes.

(d) Volcano plot showing log2 fold change vs. adjusted p-value for all proteins in area CA1 (n = 6) vs. CA3 (n = 6). Green proteins are significantly more highly expressed in area CA1 (BH adjusted p-value < 0.05, log2 FC cut-off 0.2), red proteins are significantly more highly expressed in area CA3.

2.3.2 Object recognition induces dynamic changes in protein expression that are distinct in area CA1 and CA3

To determine how learning affects the proteome in area CA1 and CA3, we trained mice on an object recognition (OR) task. On this task, each mouse is exposed to a set of 3 novel objects during a training session and is tested for memory for the objects one day later by being exposed to 2 of the previous objects and a novel one (Figure 2-2A, B). At testing, exploration of the novel object was higher than the other objects, indicating memory for the initial object (Figure 2-2C). Control animals exposed to the same set of objects during training and testing explored all objects comparably at testing, suggesting equal memory for all objects. The proteome of area CA1 and CA3 was then examined 2, 4, 8 and 24 hours after testing and compared with control mice by SWATH-MS. For area CA1 and CA3, 1420 and 1366 proteins respectively, were quantified across all time points. The level of protein expression was significantly altered in the animals exposed to a novel object at testing, in both area CA1 and CA3 and at each time point (Figure 2-2D). The number of proteins with significantly changed expression and the direction of change were however different across time points. For area CA1, 119 proteins had an altered level of expression across several time points (3 across the 4 time points, 25 across 3 time points and 90 across 2 time points). Further, 214 proteins were altered after 2 hours (95 up-regulated and 110 down-regulated), 62 proteins after 4 hours (46 up-regulated and 16 down-regulated), 168 proteins after 8 hours (124 up-regulated and 44 down-regulated) and 123 proteins after 24 hours (96 up-regulated and 27 down-regulated). For area CA3, 45 proteins were found significantly changed across several time points (2 across all 4 time points, 11 across 3 time points and 32 across 2 time points). Further, 46 proteins had changed expression after 2 hours (11 up-regulated and 35 down-regulated), 39 proteins after 4 hours (27 up-regulated and 12 down-regulated), 43 proteins after 8 hours (22 up-regulated and 21 down-regulated) and 158 proteins after 24 hours (63 up-regulated and 95 down-regulated). Thus in area CA1, many proteins were down-regulated after 2 hours then up-regulated at 8 and 24 hours. In contrast in area CA3, the most distinct changes were only after 24 hours, and earlier time points had only small changes. These results suggest that object recognition alters protein expression in a time- and hippocampus subregion-specific manner.

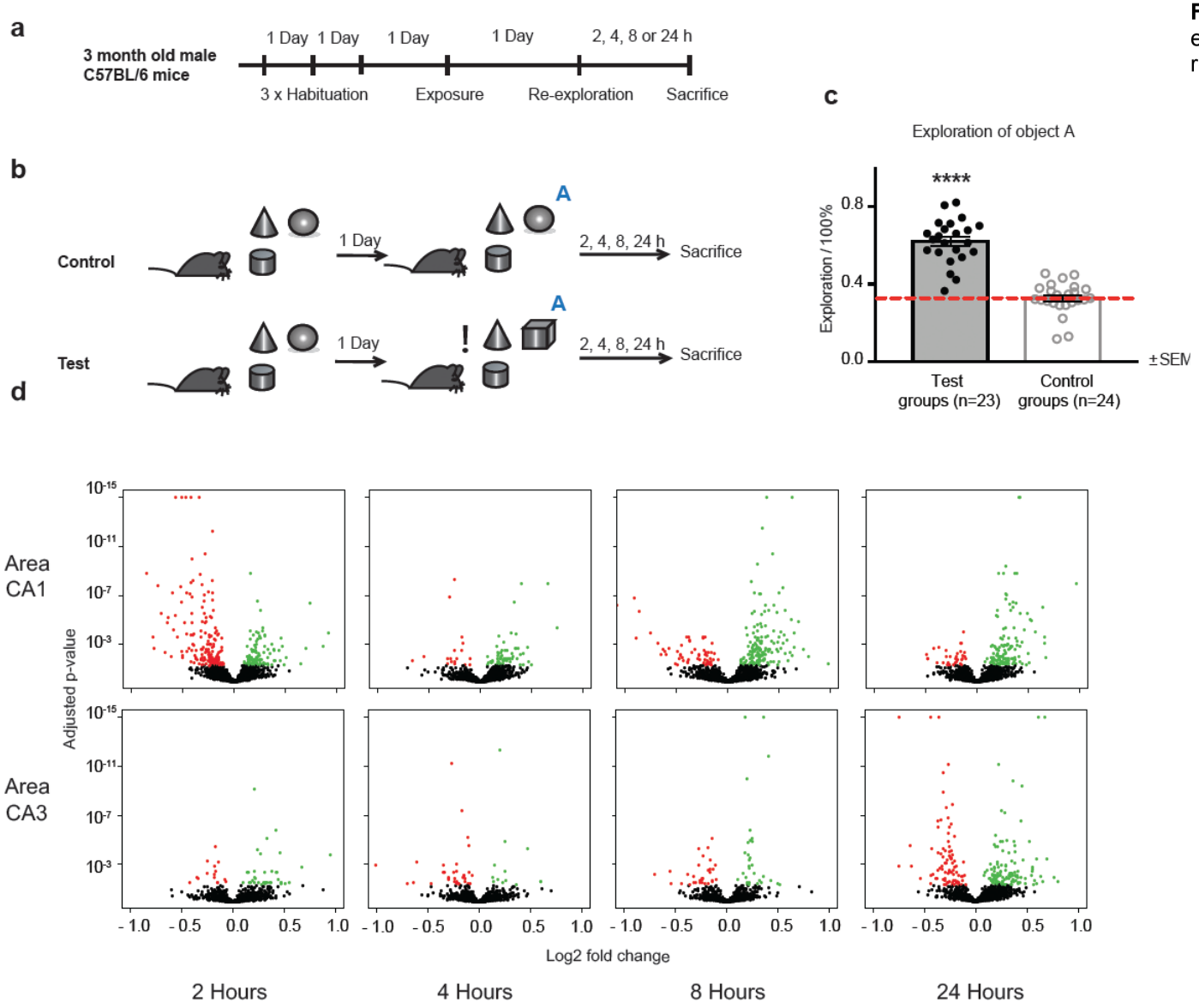


Figure 2-2: Changes in protein expression induced by object recognition (OR) in area CA1 and CA3

(a) Experimental timeline of the OR paradigm.

(b) OR exposure and test sessions for control and test animals.

(c) Exploration of object A (unfamiliar for test animals, familiar for control animals) for test and control animals (**** = P < 0.0001). The dashed red line indicates chance level exploration of 33%.

(d) Differences in protein expression in area CA1 and CA3 between test and control animals sacrificed after 2, 4, 8 or 24 hours. Green proteins are significantly more highly expressed (adj. p-value < 0.05), red proteins are significantly less expressed in test versus control samples (number of animals: Area CA1, 2h, n = 4 for each group; 4h, n = 4 for each group; 8h, n = 3 for each group; 24 h, n = 4 for each group; Area CA3, 2h, n = 3 for each group; 4h, n = 4 for each group; 8h, n = 4 for each group; 24 h, n = 3 for each group)

To examine the data in a global multi-proteins context, we searched for clusters of proteins with similar expression profile across time using a statistical clustering approach. Significant clusters of proteins could be identified in both area CA1 and CA3 (Figure 2-3A and B). We then determined if proteins within each cluster are functionally or spatially related using GO analyses for each significant cluster. Many of the significant clusters were indeed enriched for functionality or localization. Clusters with functional enrichment were identified in both area CA1 and CA3 with little overlap between the two. In area CA1, most clusters had a distinct down-regulation after 2 hours, followed by an up-regulation after 8 hours. Many of them were enriched for mitochondrial membrane and inner membrane proteins. Clusters with an opposite regulation, up-regulation after 2 hours and down-regulation after 8 hours, were also observed, and were enriched for proteins involved in the negative regulation of the MAPK cascade and regulation of GTPase/ATPase activity. In area CA3, clusters with up-regulation at both 2 and 24 hours were observed, and had poor functional enrichment. Another group of clusters had down-regulation after 2 and 24 hours, and was enriched for inner mitochondrial membrane proteins. Together, clustering analyses suggested three effects: 1) Common profiles of protein expression are enriched in area CA1 and CA3 after object recognition, 2) Proteins in a given cluster (with similar expression profile) have similar enrichment in functionality or localization, 3) Area CA1 and CA3 have distinct protein clusters and dynamics after object recognition.

a

Clusters in area CA1 after object recognition

Cluster expression profile	Cluster significance	Enrichment in functionality or localization	Adjusted p of functionality/ localization
	36 proteins assigned 4.3 expected p = 2.1 E-21	Mitochondrial inner membrane Transporter activity Electron transport chain	2.15 E-9 8.0 E-4 0.0021
	47 proteins assigned 10.1 expected p = 1.7 E-17	Miochondrial inner membrane	5.58 E-5
	24 proteins assigned 4.4 expecte p = 5.5 E-11	Membrane proteins Mitochondrial membrane Ionotropic glutamate receptor complex	1.87 E-5 0.0057 0.0021
	17 proteins assigned 3.1 expected p = 2.9 E-8	No enrichment	
	28 proteins assigned 6.3 expected p = 1.6 E-10	Negative regulation of MAPK cascade Negulation of GTPase activity Actin dependent ATPase activity	0.0041 0.0476 0.0190
	26 proteins assigned 8.6 expected p = 1.2 E-6	Threonine endopeptidase activity	0.0280
	21 proteins assigned 6.7 expected p = 6.6 E-6	Oxoglutamate dehydrogenase complex	0.0396
	17 proteins assigned 4.6 expected p = 7.2 E-6	Ionotropic glutamate receptor binding NADH dehydrogenase activity	0.0467 0.0477

b

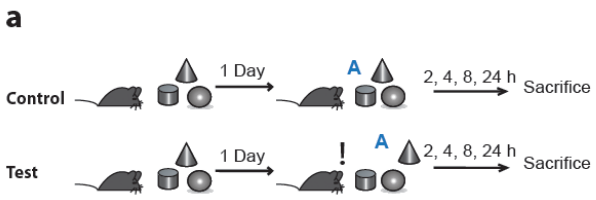
Clusters in area CA3 after object recognition

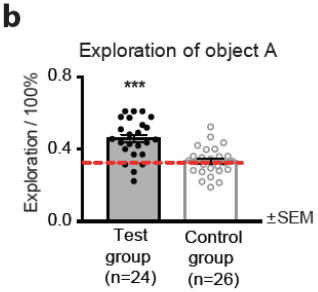
Cluster expression profile	Cluster significance	Enrichment in functionality or localization	Adjusted p of functionality/ localization
	32 proteins assigned 3.5 expected p = 2.7 E-20	Double-strand break repair	0.0357
	32 proteins assigned 5.3 expected p = 2.4 E-15	No enrichment	
	27 proteins assigned 6.6 expected p = 1.6 E-9	No enrichment	
	19 proteins assigned 3.9 expected p = 3.0 E-8	Aminoacyl-tRNA ligase activity	0.0132
	13 proteins assigned 2.8 expected p = 8.0 E-6	GTP binding GTPase activity Tubulin complex	0.0321 0.0398 0.0123
	15 proteins assigned 4.6 expected p = 1.9 E-8	Negative regulation of ERK 1/2 cascades Regulation of ROS metabolic process	0.0374 0.0374
	21 proteins assigned 6.4 expected p = 2.2 E-10	Mitochondrial inner membrane Transmembrane transport	3.65 E-12 0.0460
	14 proteins assigned 2.3 expected p = 1.8 E-7	Mitochondrial inner membrane Respiratory chain	6.61 E-5 9.3 E-5
	38 proteins assigned 12.9 expected p = 8.3 E-9	No enrichment	

Figure 2-3: Enriched protein expression profiles in area CA1 (a) and CA3 (b) following OR. Profile shapes over time are shown in the left column. Other columns (from left to right) show cluster significance determined by STEM following multiple testing corrections, enrichment in functionality or localization within profiles determined by GO analyses and significance of GO analyses enrichments.

2.3.3 Recognition of object location alters protein expression in area CA1 and CA3 differently to recognition of object

To investigate if protein expression in area CA1 and CA3 is changed in a common or task-specific manner, we repeated the experiments using a second learning paradigm related to object recognition but different, an object location recognition (OLR) task (Figure 2-4A). On this task, animals have to remember the location of an object (tested by displacing the object and assessing exploration after displacement) in an arena containing three familiar objects. OLR is known to specifically recruit hippocampal functions, while OR relies also on cortical structures. Changes in protein expression across time were examined as with OR by SWATH-MS (Figure S2) followed by statistical clustering. Again, significant clusters of proteins in both area CA1 and CA3 could be detected (Figure 2-4C and D). In area CA1, clusters with down-regulation after 2 hours and up-regulation after 8 hours and enriched for mitochondrial inner membrane proteins were observed, similarly to the OR paradigm. However in area CA3, only clusters with a distinct up-regulation after 4 hours and enriched for synaptic proteins were detected. These results suggest that object location recognition induces changes in protein expression in a time- and hippocampus subregion-specific manner.





c

Clusters in area CA1 after object location recognition

Cluster expression profile	Cluster significance	Enrichment in functionality or localization	Adjusted p of functionality/ localization
	20 proteins assigned 2.6 expected p = 5.4 E-12	Mitochondiral inner membrane Mitochondrial membrane Cytochrome-c oxidase activity	2.83 E-6 3.0 E-4 0.0180
	23 proteins assigned 4.4 expected p = 2.5 E-10	Mitochondrial inner membrane Voltage-gated anion channel activity Transport	2.62 E-7 0.0270 0.0330
	18 proteins assigned 4.2 expected p = 4.2 E-7	No enrichment	

d

Clusters in area CA3 after object location recognition

Cluster expression profile	Cluster significance	Enrichment in functionality or localization	Adjusted p of functionality/ localization
	43 proteins assigned 8 expected p = 9.9 E-19	Synapse Cytoplasmic vesicles Synaptic vesicle membrane	0.0004 0.0032 0.0045
	25 proteins assigned 3.3 expected p = 1.2 E-14	Protein phosphatase type 2A regulator activity	0.0320
	13 proteins assigned 1.5 expected p = 7.7 E-9	Positive regulation of signaling Positive regulation of glucose transport Positive regulation of FGFR signaling pathway	0.0310 0.0255 0.0127
	13 proteins assigned 1.9 expected p = 8.9 E-8	No enrichment	
	10 proteins assigned 1.3 expected p = 9.3 E-7	No enrichment	
	25 proteins assigned 3.3 expected p = 1.2 E-14	Protein phosphatase type 2A regulator activity	0.0320
	15 proteins assigned 1.8 expected p = 6.6 E-10	Stress fiber	0.0285

Figure 2-4: Changes in protein expression induced by OLR in area CA1 and CA3

(a) OR exposure and test sessions for control and test animals

(b) Exploration of object A (displaced for test animals, not displaced for control animals) for test and control animals (*** = P < 0.001). The dashed red line indicates chance level exploration of 33%.

(c) Enriched protein expression profiles in area CA1 (c) and CA3 (d) following OLR. Profile shapes over time are shown in the left column. Other columns (from left to right) show cluster significance determined by STEM following multiple testing corrections, enrichment in functionality or localization within profiles determined by GO analyses and significance of GO analyses enrichments. (number of animals: Area CA1, 2h, n = 4 for each group; 4h, n = 4 for each group; 8h, n = 4 for control and n = 3 for test; 24 h, n = 4 for each group; Area CA3, 2h, n = 4 for each group; 4h, n = 4 for each group; 8h, n = 4 for each group; 24 h, n = 4 for each group)

2.3.4 Changes in protein expression after OR and OLR correlate in area CA1 but not in area CA3

To assess the correlation between OR and OLR paradigms, we compared the expression patterns of protein clusters across the 2 paradigms (Figure 2-5A and B). Comparing cross-correlations between data sets revealed decreased p-values for clusters observed in both OR and OLR in area CA1 (Figure 2-5C). In contrast, there was no increase in correlation in area CA3 between the two paradigms, or between area CA1 and CA3 within paradigms when compared with randomized data (Figure 2-5D). Overall, these analyses thus revealed that 1) changes in protein expression induced by training are different in area CA1 and CA3, 2) changes in area CA1 correlate in both OR and OLR tasks, and 3) changes in area CA3 do not correlate between OR and OLR tasks. These results suggest that proteome-wise, area CA1 contributes to object and object location recognition in a similar manner, while area CA3 contributes differently.

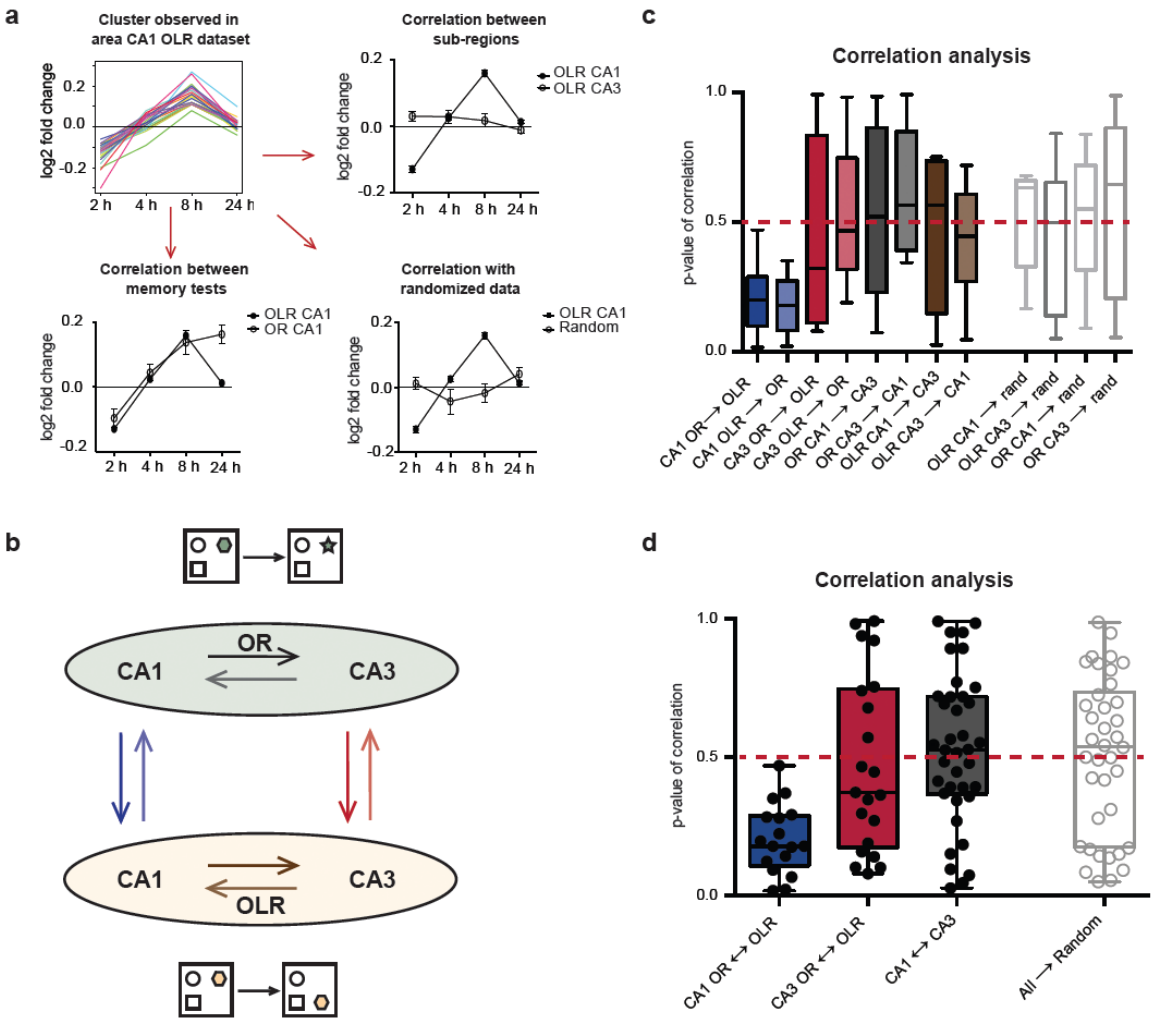


Figure 2-5: Correlation of changes in protein expression between OR and OLR and area CA1 and CA3

(a) Correlation analyses for one illustrative cluster. Top left: expression change data of all proteins within this cluster in the OLR paradigm in area CA1 between control and test at each time-point. Top right: Expression change of the same proteins in OLR area CA1 and OLR area CA3 shows correlation between sub-regions for these proteins. Bottom left:, Expression change of the same proteins in OLR area CA1 and OR area CA1 shows correlation between memory tests for these proteins. Bottom right: correlation with a randomized data set (random re-assignment of fold-changes within the experiment).

(b) Illustration of analyzed correlations between OR and OLR and between area CA1 and CA3.

(c) P-values of correlation for comparisons in (b) (kruksal-wallis p-value = 0.0249, column statistics with wilcoxn signed rank test and hypothetical mean of 0.5: CA1 OR ➔ OLR p = 0.0005, CA1 OLR ➔ OR p = 0.0625), show increased correlation within area CA1 between learning paradigms. Colors of boxes correspond to colors of arrows in (b).

(d) Pooled data for (c) combining CA1 OR vs. OLR correlations (blue in (b)), CA3 OR vs. OLR correlations (red in (b)), correlations of CA1 vs. CA3 in both paradigms (gray and brown in (b)) and all correlations with randomized data (white in (b)). (kruksal-wallis p-value = 0.0008, column statistics with wilcoxn signed rank test and hypothetical mean of 0.5: CA1 OR ↔ OLR $p < 0.0001$, all other ns.)

2.3.5 Electron transport chain proteins are selectively regulated in area CA1 following object and object location recognition

To gain functional insight into the proteins activated in the clusters, we searched for proteins enriched in area CA1 after both, object or object location recognition. Many of the proteins within these clusters are located in the inner mitochondrial membrane, and are components of the electron transport chain (ETC). Notably, all ETC proteins assigned to significant clusters had similar dynamics, they were down-regulated after 2 hours then up-regulated after 8 hours (Figure 6A). Up-regulation after 8 hours was validated for ATP5A with new biological replicates, including a third no re-exposure control group, confirming an OR specific effect of ATP5A (Figure 2-S5). Correlation analyses of all quantifiable ETC proteins revealed a strong correlation between OR and OLR in area CA1 but not in area CA3 (Figure 2-6B and C). Further, comparing ETC proteins with other mitochondrial proteins showed that changes are specific for ETC proteins and do not affect other mitochondrial proteins (Figure 6D).

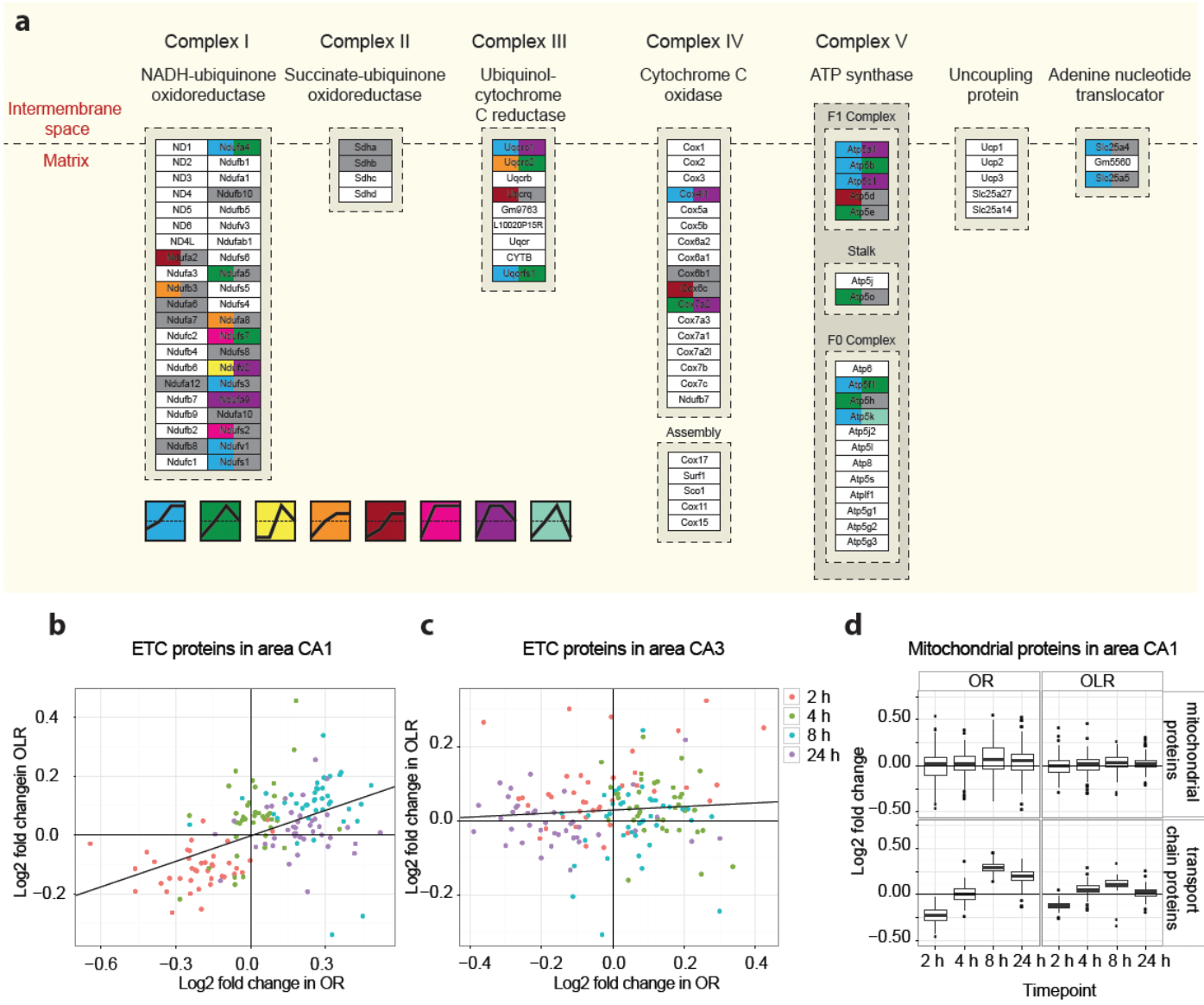


Figure 2-6: Changes in electron transport chain (ETC) proteins following OR and OLR.

(a) Expression profile of ETC proteins in area CA1 following OR and OLR. White protein boxes indicate proteins that could not be quantified. Color on the left side of protein boxes indicates their expression profile in the OR paradigm, color on the right side indicates expression profile in the OLR paradigm. Grey color indicates proteins that did not follow any of the colored profiles.

(b) Linear regression model of all quantifiable ETC protein log2 fold changes between OR and OLR in area CA1. Colors indicate time-points. Linear model statistics: log2OR = 0.29 * log2OLR, p-value of slope = $3.19*10^{-12}$, R^2 = 0.27;

(c) Linear regression model of all quantifiable ETC protein fold changes between OR and OLR in area CA3 shows no significant correlation.

(d) Boxplot displaying log2 fold changes of ETC protein and all other mitochondrial proteins in area CA1 following both OR and OLR.

2.4 Discussion

This study provides evidence that different forms of learning activate different and specific proteomes in hippocampus area CA1 and CA3. It shows that recognition of an object or recognition of an object location induces changes in protein expression that distinguish area CA1 and CA3, and are dynamically regulated differently in these areas across time. It identifies clusters of proteins within a sub-region that are enriched for functions and cellular distribution, and have similar expression profiles after both tasks. Comparing the type, expression pattern and temporal regulation of proteins represented in each cluster reveals a strong correlation between OR and OLR paradigms in area CA1 but not in area CA3, suggesting striking differences between the proteome of area CA1 and CA3 relevant for memory formation.

The hippocampus is a key brain structure for cognitive processes that allows the formation of episodic memory and the detection of novelty. The formation of episodic memory requires the encoding of contexts and information encountered at a given time, as well as the encoding of the temporal relationship of the different contexts, for instance the order in which they were encountered. The detection of novelty by the hippocampus involves the recall of stored memories about context and the identification of match/mismatch with the newly encountered context. Area CA1 and CA3 contribute differently to these processes. While area CA1 is necessary for the detection of novelty (Lisman and Otmakhova 2001) and the encoding of the temporal order of contexts (Huerta, Sun et al. 2000, Gilbert, Kesner et al. 2001, Hoge and Kesner 2007, Hunsaker, Lee et al. 2008, Kesner, Hunsaker et al. 2010), area CA3 is required for the encoding of the context itself (Leutgeb, Leutgeb et al. 2004, Rajji, Chapman et al. 2006). Our results identify a pool of proteins whose expression is changed in area CA1 after two behavioral tasks that rely upon the detection of novelty, novelty for an object in OR and novelty for an object location in OLR. These proteomic changes are likely associated with the detection of novelty because they are not induced when the same object or the same location of object are presented. They may be necessary for the formation of new episodic memory. In contrast to area CA1, changes in protein expression in area CA3 are different after OR and OLR. While only few

proteins are changed following OR, many proteins, in particular synaptic proteins, are up-regulated 4 hours after OLR. This suggests that different processes are engaged in area CA3 after OR and OLR, that may depend on the type of novelty. This is consistent with the role that area CA3 plays in the encoding of context, which in the OLR paradigm, involves a change in location (spatial configuration) of an object. It has been proposed that a context can have both spatial and non-spatial components, and that spatial components are encoded first to generate a 'scaffold' for representation of the whole context. Non-spatial cues are then added to this scaffold in subsequent steps (Knierim, Lee et al. 2006). This implies that alteration of spatial features of a context, like occurring in OLR but not OR, would require either the remodeling of an existing neural representation of the context, or the formation of a new representation. Such process would likely rely on plasticity within area CA3, a process that may require *de novo* protein synthesis (Cajigas, Will et al. 2010) as exemplified by the changes in synaptic proteins observed in our proteomic analyses.

Another striking feature of our data is the temporal alteration of the expression of ETC proteins in area CA1 following both, OR and OLR. Such alteration could be induced by the detection of novelty and contribute to neuronal activity necessary for the formation of episodic memory. The recognition of novelty in OR and OLR has been associated with changes in activity in area CA1 but not CA3 (Larkin, Lykken et al. 2014); it increases the activity of excitatory CA1 pyramidal neurons but decreases the activity of inhibitory CA1 interneurons (Wilson and McNaughton 1993, Nitz and McNaughton 2004, Csicsvari, O'Neill et al. 2007, Karlsson and Frank 2008, VanElzakker, Fevurly et al. 2008). Neurons in area CA1 are not only more readily activated but their increased excitability lasts longer (Moyer, Thompson et al. 1996, McKay, Matthews et al. 2009). The increased excitability could result from higher intracellular ATP, which in neurons, attenuates K_{ATP} channels, favors membrane depolarization and shifts cells into a more excitable state (Huang, Huang et al. 2007). ATP itself is produced at the inner mitochondrial membrane by ETC via oxidative phosphorylation. Thus, the increase in ETC proteins observed in area CA1, but not area CA3, may elevate neuronal ATP production 8 and 24 hours after training, and thereby sustain persistent excitability specifically in these neurons. This may ultimately help the formation of episodic

memory since increased excitability in area CA1 has been shown to be linked to episodic memory formation (Cai, Aharoni et al. 2016). The ensemble of neurons in area CA1 that are activated during contextual tasks have increased excitability so as to facilitate the concurrent encoding of other contextual memories occurring within close temporal proximity. Several studies confirmed that area CA1 is key to form temporal associations between different contexts (Huerta, Sun et al. 2000, Gilbert, Kesner et al. 2001, Hoge and Kesner 2007, Hunsaker, Lee et al. 2008, Kesner, Hunsaker et al. 2010).

Multiple links between ETC proteins and memory formation have previously been reported in aging and pathological conditions. Decreased excitability of CA1 pyramidal neurons was demonstrated in aged mice and contributes to aging-related cognitive impairments (Kaczorowski and Disterhoft 2009, Oh, Oliveira et al. 2010, Oh, Simkin et al. 2016). Aging-related mitochondrial dysfunctions, including reduced ETC efficiency, have also been described in mice (Navarro, Lopez-Cepero et al. 2008, Navarro and Boveris 2010), providing a link between ETC functions and aging-related decrease of area CA1 neuron excitability. Similar mitochondrial dysfunctions have also been observed in neurodegenerative disorders characterized by cognitive impairment such as Alzheimer's disease (Maruszak and Zekanowski 2011), Parkinson's disease (Winklhofer and Haass 2010), traumatic stress (Zhang, Zhou et al. 2006), major depressive disorder (Tobe 2013) and hypoxia (Solaini, Baracca et al. 2010).

Intriguingly, while ETC proteins were increased 8 and 24 hours after novelty detection, they were decreased 2 hours after, suggesting dynamic regulation of these proteins and different functions at a short- and long-term. Although an increased and prolonged excitability of CA1 neurons could help memory formation, it may also make neurons more susceptible to oxidative damage, in particular, following periods of high activity. Oxidative damage occurs when the production of reactive oxygen species (ROS) is excessive and cannot be overcome by the ability of the cell to chemically deactivate them through reduction. Most ROS are produced in mitochondria, predominantly in complexes I and III of the ETC, and are present as a natural by-product of cellular metabolism (Lambert and Brand 2009). Neuronal stimulation through NMDA receptors increases ROS production,

and overstimulation can damage neurons and lead to excitotoxic cell death (Gunasekar, Kanthasamy et al. 1995, Girouard, Wang et al. 2009). Down-regulation of ETC proteins in area CA1 2 hours after training may be a protective mechanism to prevent oxidative damage following a period of increased activity. Such down-regulation could occur by directed degradation of ETC proteins through the ubiquitin-proteasome system (UPS), shown to control multiple aspects of mitochondrial functions (Franz, Kevei et al. 2015). It can selectively tag proteins by conjugation with ubiquitin followed by degradation (Nandi, Tahiliani et al. 2006). Many inner mitochondrial proteins can be conjugated to ubiquitin and ubiquitin system components localize to mitochondria (Lehmann, Udasin et al. 2016), allowing the UPS to potentially function within mitochondria.

The present data therefore suggest that a dynamic system through which ETC proteins are temporally regulated may have a key role in balancing cell excitability within area CA1 of the hippocampus. Impairments in this system could contribute to pathologies characterized by anomalies in ETC expression. Better understanding the dynamics and regulation of ETC proteins could provide means for novel therapeutic approaches in these pathologies.

2.5 Procedures

For an overview of the sample processing and data analysis workflow see Figure 2S-6.

2.5.1 Animal care and housing

Care of animals and all protocols conformed to the guidelines of the Veterinary Office of the Canton of Zurich, Switzerland, and approved by the Commission for Animal Research (54/2012 and 41/2015). C57BL/6 males were maintained in a temperature- and humidity-controlled facility on a 12h-reversed light/dark cycle with food and water *ad libitum in cages of 3-5 animals*. Subjects were euthanized by cervical dislocation.

2.5.2 Object recognition (OR) and object location recognition (OLR) paradigm

OR and OLR were used to induce memory for objects or object location in adult (2-3 months old) wildtype C57BL/6 male mice. Animals were habituated to an empty arena (square box with plexiglas walls (40 x 40 x 40 cm)) in a dimly lit room. Each animal was allowed to explore the arena individually for 5 minutes. Habituation was repeated daily on three consecutive days. For training, animals were placed in the arena containing three different unfamiliar objects located on 3 of the 4 corners of a virtual square in the center (20 x 20 cm) of the arena. The animals explored the set of objects for 15 minutes. Exploration of the objects was recorded with an infrared camera and analyzed with a videotracking system (Viewpoint Behavior Technology, Lyon, FR). Animals were returned to their home cage for 24 hours before testing. In OR, animals were tested with two of the initial objects and a novel object (unfamiliar to the animals) for 15 mins. In OLR, animals were tested with the three familiar objects but one was spatially displaced (moved to the empty corner of the square in the center) for 15 mins. Control animals were tested with the same objects at the same place. Exploration of objects was also tracked manually using Labwatcher (Viewpoint Behavior Technology, Lyon, FR). Animals were sacrificed 2, 4, 8 or 24 hours after testing.

2.5.3 Experimental design

Animals from the same cage were evenly distributed to test and control groups for each time point (2, 4, 8 and 24 hours). Behavioral testing was conducted in successive sessions, each session with 6 test and 6 control animals at a given time point. Behavioral sessions always started at the same time of the day (11:00 am). A block design was used to reduce bias from samples processing or measurement. For this, samples were divided into multiple blocks, with each block containing one sample of each group (test and control) at each time point. The order in which blocks were processed and measured was randomized. The sample order within blocks was first randomized for sample processing (protein extraction, protein digestion and peptidic clean-up) and then re-randomized for SWATH-MS measurements.

2.5.4 Dissection of area CA1 and CA3

Animals were killed by cervical dislocation followed by decapitation. Their brain was isolated in a tray of ice-cold PBS buffer and transferred to filter paper for dissecting the hippocampus. Filter papers were cooled with PBS-ice to prevent tissue warming. Isolated hippocampi were transferred to a binocular microscope and cooled with PBS-ice. Area CA3 was dissected by cutting along the minor hippocampal fissure along the dorso-ventral axis of the hippocampus. Area CA1 and dentate gyrus were separated with a pincer and incision scalpel by gently pushing the blade along the major hippocampal fissure. Area CA1 and CA3 were snap frozen in liquid-nitrogen and stored at -80°C until further processing.

2.5.5 Protein extraction and digestion

Proteins were extracted and processed for SWATH-MS analyses. Proteins were extracted from area CA1 and CA3 samples using 200ul TEAB buffer (100mM triethylammonium bicarbonate, 0.1% SDS, 1:100 protease inhibitor cocktail P8340 (Sigma-Aldrich, St. Louis MO, USA), 1:500 PMSF (50mM in EtOH)). The samples were mechanically lysed by 15 strokes with a 26G needle and sonicated for 2 minutes. Samples were spun down at 16000g for 30 minutes at 4°C and supernatants were collected. Proteins were quantified using a Qubit protein assay kit (ThermoFisher Scientific, Waltham MA, USA) following the manufacturer's

protocol. Protein extracts were further processed with a filter assisted sample preparation protocol (Wisniewski, Zougman et al. 2009). 20ug of protein were filled up to 30ul of final volume with SDS denaturation buffer (4% SDS (w/v), 100mM Tris/HCL pH 8.2, 0.1M DTT). For denaturation, samples were incubated at 95°C for 5 min. Samples were diluted with 200ul UA buffer (8M urea, 100mM Tris/HCl pH 8.2) and then loaded to regenerated cellulose centrifugal filter units (Microcon 30, Merck Millipore, Billercia MA, USA). Samples were spun at 14000g at 35°C for 20 min. Filter units were washed once with 200ul of UA buffer followed by centrifugation 14000g at 35°C for 20 min. Cysteines were blocked with 100ul IAA solution (0.05M iodoacetamide in UA buffer) for 1 min at room temperature in a thermomixer at 600rpm followed by centrifugation at 14000g at 35°C for 15 min. Filter units were washed 3 times with 100ul of UA buffer then twice with a 0.5M NaCl solution in water (each washing was followed by centrifugation at 35°C and 14000g for 15 min). Proteins were digested overnight at room temperature with a 1:50 ratio of trypsin (0.4ug) in 130ul TEAB (0.05M Triethylammoniumbicarbonate in water). After protein digestion, peptide solutions were spun down at 14000g at 35°C for 15 min and acidified with 3ul of 20% TFA (trifluoroacetic acid).

2.5.6 Hydrophobic interaction liquid chromatography for peptide fractionation

HILIC (hydrophobic interaction chromatography) was used to fractionate peptides from whole hippocampus protein extracts in order to prepare a broader spectral library. The trypsinated sample was filled up to a final volume of 1.3 ml of 75% ACN, 10 mM KH_2PO_4. 5ul of 50% phosphoric acid was added to ensure a pH of 4.5. The sample was fractionated using an 1100 series HPLC system (Agilent, Santa Clara, CA) into 9 separate fractions.

2.5.7 Peptides clean-up

Peptides were cleaned-up using Sep-Pak C18 silica columns (Waters Corporation, Milford MA, USA). Columns were activated with 1ml methanol and washed with 1ml of 60% ACN (acetonitrile) and 0.1% TFA. Columns were equilibrated with 3 x 1ml of 3% ACN 0,1% TFA. Samples were diluted in 800ul of 3% ACN 0.1% TFA and loaded onto the columns. They were then washed with 4x 1ml 3% ACN 0.1%

TFA and eluted with 60% ACN 0.1% TFA. Samples were lyophilized in a speedvac then re-solubilized in 19ul 3% ACN 0.1% FA (formic acid) prior to measurement. 1ul of synthetic peptides (Biognosys AG, Switzerland) were added to each sample for retention time calibration.

2.5.8 SWATH-MS measurements

Samples were measured on a TripleTOF 5600 (AB Sciex, Washington, USA) in SWATH mode using a previously described method (Gillet, Navarro et al. 2012). Peptides were separated with an Eksigent NanoLC (AB Sciex, Washington, USA). 4ul of each sample were injected and loaded onto a 1.8 um, 100 Å C18 column (heated to 50°C). Peptides were separated using a 144 min linear solvent gradient of 3-40% ACN. Fixed 25Da precursor isolation windows were used within a precursor range of 300-1200 m/z. Fragment ions were acquired in a range of 200-1800 m/z.

2.5.9 Shotgun measurements

To generate an extensive tissue-specific spectral library, samples from the SWATH-MS sample pool and fractions from pre-fractioned hippocampus sample were used for shotgun measurements. 8 samples from the sample pool of both OR and OLR experiments were chosen randomly. Additionally, all nine HILIC fractions from whole hippocampus extracts were individually measured. Measurements were performed on a TripleTOF 5600 System (AB Sciex, Washington, USA) as for SWATH-MS measurements. Peptides were separated with an Eksigent NanoLC (AB Sciex, Washington, USA). 4ul of each sample were injected and loaded onto a 1.8 um, 100 Å C18 column (heated to 50°C). Peptides were separated with using a 144 min long linear solvent gradient of 3-40% ACN. MS1 were acquired in a 300-1200 m/z range, MS2 in a 200-1800 m/z range.

2.5.10 MS/MS ion searches of shotgun measurements

MASCOT (Matrix Science Ltd, London, GB) was used for MS/MS ion searches. For each shotgun run a Mascot search file was generated. Mascot search files from all shotgun runs were merged into a single search file using mascot daemon (Matrix Science Ltd, London, GB). The merge file was searched against a tryptic

digestion of a decoyed mouse proteome (Uniprot taxon identifier 10090). Carbamidomethyl at cysteine was assumed as fixed modification and oxidation of methionine as variable modification. A peptide mass tolerance of +/- 20ppm and a fragment mass tolerance of +/-0.1 Da was used.

2.5.11 Generation of a spectral library and a precursor-iRT list

DAT files from MS/MS ion searches were exported with Mascot and loaded into Skyline (MacCoss Lab) to generate a spectral library with a false discovery rate of 1%. Libraries were generated with single entries for every precursor, choosing the ones with the best mascot scores if multiple spectra were available. Retention times of spiked in iRT-peptides (Biognosys AG, Switzerland) from all shotgun MS runs were manually exported and used to generate an iRT versus retention time linear regression for every run. These regressions were then used to calculate iRT values for all spectral library entries from the measured retention times of the corresponding MS runs. Using this approach, a precursor list with corrected iRT values for the whole proteome was generated.

2.5.12 Generation of a Spectronaut assay list

The precursor-iRT list and the spectral library were used to generate an assay list for Spectronaut (version 6, Biognosys AG, Switzerland). The whole mouse proteome from Uniprot (Taxonom identifier 10090) was loaded into Skyline and an *in silico* digestion using trypsin with a KR|P cutting profile without allowing for any missed cleavage was performed. Peptides with a minimum length of 8 and a maximum length of 25 were included and 25 N-terminal amino acids were excluded. Carbamidomethyl was assumed as a fixed modification for cysteine. Precursor charges of 2+ or 3+ and ion charges of 1+ or 2+ for all ion types (x,y,z,a,b,c) were included. For each precursor, the 4 fragment ions with the highest intensity within an m/z range of 200-1800 in the spectral library were selected and exported as assay list compatible with Spectronaut (version 6, Biognosys AG, Switzerland). Correct iRT values were added to the assay list by comparison with the precursor-iRT list.

2.5.13 SWATH-MS transition identification and quantification

For identification and quantification of transitions in SWATH-MS data (Gillet, Navarro et al. 2012), automated peak picking was used. The previously prepared assay list was used to define transitions, relative intensities and iRT values. Spectronaut (version 6, Biognosys AG, Switzerland) was used for automated peak picking. Peak picking was performed independently for area CA1 and CA3 and independently for OR and OLR paradigms. A static window with an iRT-width of 6 min and a linear iRT calibration was used. For transition identification, a dynamic score refinement was used and the base entity was processed within the experiment. Decoys were generated using a scrambled, label-free decoy method. A normal distribution estimator was used for q-values. For transition quantification an interference correction was used and the total peak area was used as normalization base.

2.5.14 SWATH-MS data normalization

SWATH-MS data was normalized with a custom script (Script 1). Since large changes in time-dependent intensity were observed over different MS runs, a time-dependent normalization approach was used. Transitions were divided into 15 time-windows according to their predicted iRT values. Time-windows were normalized independently by summing up all intensities within MS runs and calculating a normalization factor matrix for all MS runs and time windows. Intensities for all transitions were then multiplied with the corresponding normalization factor.

2.5.15 Data analyses

Following normalization, other scripts (Script 2-4) were used to assign group and condition to each run and have a q-value cutoff over the experiments. Transitions for individual samples with a q-value > 0.01 and transitions for all samples with a q-value > 0.01 in more than 25% of samples were excluded. Further, proteins quantified with less than 2 peptides after q-value cutoff were excluded. Duplicate protein entries were removed before running any statistical analysis. For statistical analyses, MSstats (Choi, Chang et al. 2014) was used, a proteomic tool using an R interface for statistical analysis of proteomic data on the transition level. MSstats

perform an ANOVA over the whole data set followed by two-group comparisons. Analyses were performed independently for area CA1 and CA3, and for OR and OLR paradigms. Group comparisons between area CA1 and CA3 in basal conditions, and group comparisons between control-test pairs for every time point were performed.

2.5.16 Statistical clustering of fold change

Short time expression miner (STEM) software (v 1.3.8) (Ernst and Bar-Joseph 2006) was used for statistical clustering of fold change values over time. For each combination of paradigm and subregion, a text file was prepared containing each protein with log2 fold change values of control-test comparison at each of the 4 time points. No normalization was performed with STEM software and a default STEM clustering method was used. 200 model profiles were generated with a maximum unit change in model profiles between time-points of 2. The minimum absolute expression change was set to 0.2 for maximum-minimum. For all other parameters, default values were used.

2.5.17 Functional annotation of statistical clusters

For each significant cluster, a list of swissprot accessions was extracted. The web-based gene set analysis toolkit (Zhang, Kirov et al. 2005) (webgestalt.org) was used for functional annotation. The protein list of each cluster was uploaded together with a list containing background identifiers from the same experiment. A GO analysis was performed using a hypergeometric statistical method with a BH adjustment for multiple testing. Minimum number of genes for a category was set to 2.

2.6 Acknowledgements

This work was supported by the University of Zurich, the Swiss Federal Institute of Technology, the Swiss National Science Foundation, and the Functional Genomics Center Zürich. We thank Prof. Ralph Schlapbach for scientific coordination at FGCZ, Asa Wahlander, Bernd Roschitzcki, Christian Trachsel and Claudia Fortes for technical assistance and Jonas Grossmann and Christian Panse for IT assistance.

2.7 Author contributions

IMM and LvZ conceived the project and wrote the manuscript. LvZ performed experiments and analyzed the data. NS contributed technical and theoretical expertise for proteomic measurements and data analyses. EK and RTC performed some behavioral experiments.

2.8 Supplementary Figures

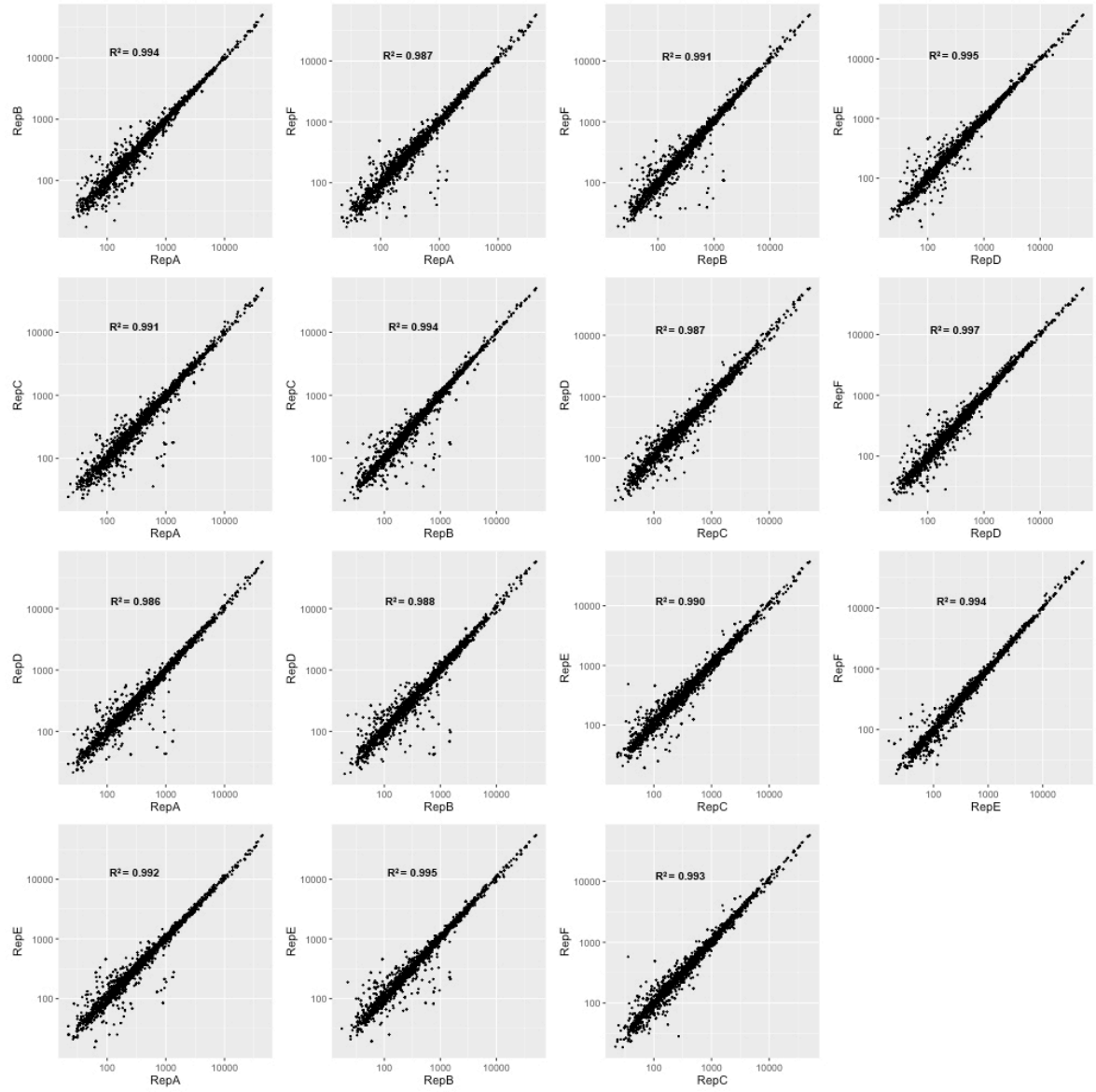


Figure 2-S1: Pearson correlations between measured protein intensity (= summed up fragment intensity of all corresponding peptides) with SWATH-MS in area CA1 of 6 biological replicates at baseline.

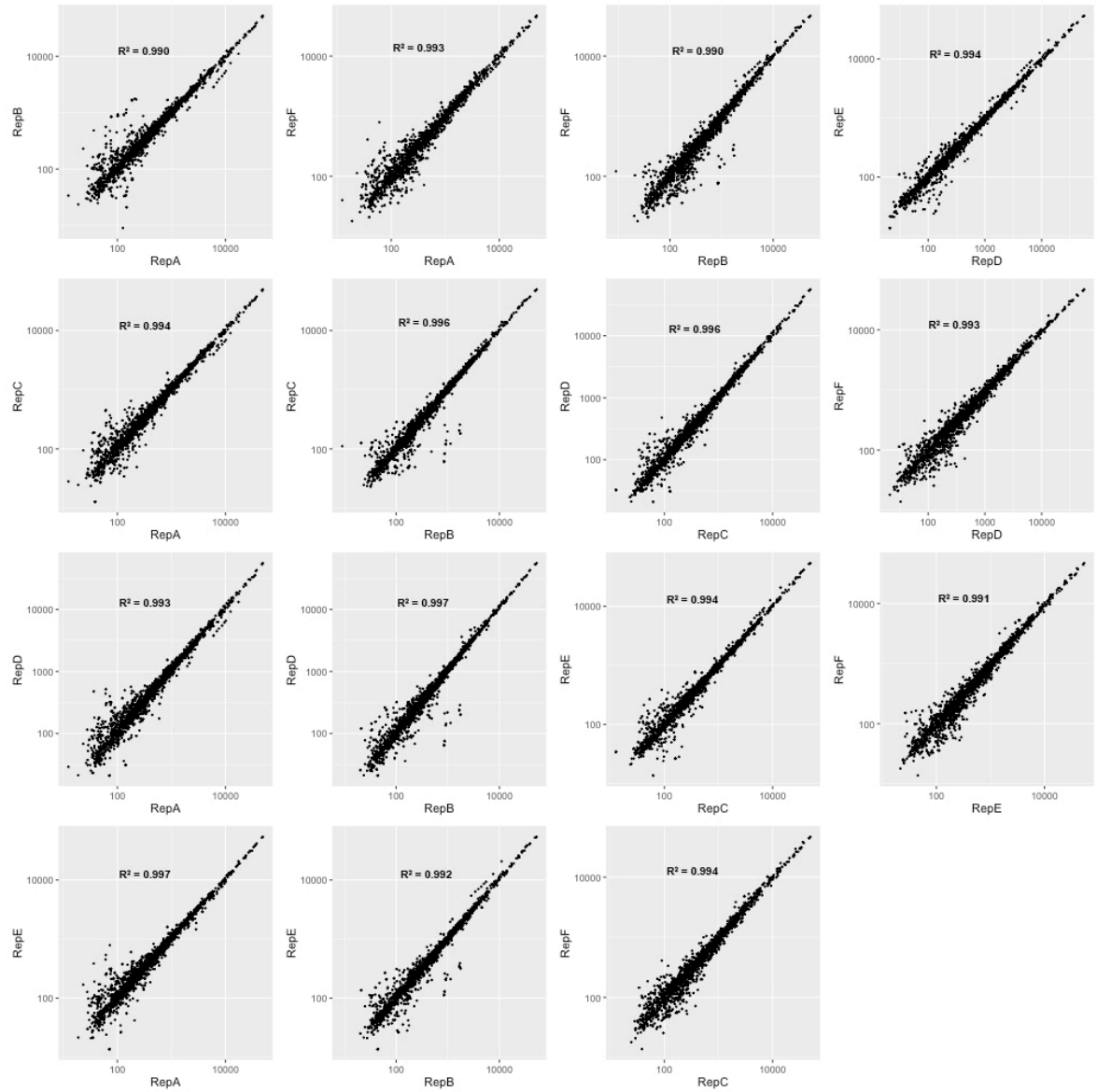


Figure 2-S2: Pearson correlations between measured protein intensity (= summed up fragment intensity of all corresponding peptides) with SWATH-MS in area CA1 of 6 biological replicates at baseline.

Full name	Short name	mRNA CA3/CA1	Protein CA3/CA1
Copine-4	Cpne4	3.98	0.67
Neurocalcin-delta	Ncald	3.33	0.40
Synaptoporin	Synpr	2.69	0.63
Hippocalcin-like protein 1	Hpcal1	1.89	0.50
Rabphilin-3A	Rph3a	1.85	-0.25
Protein piccolo	Pclo	1.60	-0.28
Hexokinase-2	Hk2	1.49	0.29
Stathmin-2	Stmn2	1.35	-0.38
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	Dlat	1.00	0.27
CD200 antigen	Cd200	0.84	-0.29
Mammalian ependymin-related protein 1	Epdr1	0.79	0.23
Annexin A6	Anxa6	0.78	0.26
Kinesin light chain 1	Klc1	0.76	-0.21
Glutathione S-transferase A4	Gsta4	0.68	-0.24
Mitochondrial glutamate carrier 1	Slc25a22	0.63	0.42
Importin subunit alpha-7	Kpna6	-0.42	0.24
EF-hand domain-containing protein D2	Efhd2	-0.62	-0.61
L-lactate dehydrogenase B chain	Ldhb	-0.67	-0.41
Protein IMPACT	Impact	-0.71	-0.20
Septin-9	Sept9	-0.76	-0.26
Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform	Ppp2r2b	-0.76	-0.25
Thioredoxin-dependent peroxide reductase, mitochondrial	Prdx3	-0.79	-0.23
Catenin delta-2	Ctnnd2	-0.79	-0.20
Neuromodulin	Gap43	-0.81	-0.70
Protein FAM49B	Fam49b	-0.86	-0.34
OCIA domain-containing protein 2	Ociad2	-0.97	0.34
Protein FAM49A	Fam49a	-1.00	-0.35
RasGAP-activating-like protein 1	Rasal1	-1.15	-0.43
Aldehyde dehydrogenase, mitochondrial	Aldh2	-1.15	-0.40
N-terminal EF-hand calcium-binding protein 2	Necab2	-2.00	0.26
Neurotrimin	Ntm	-2.12	-0.61
Alpha globin 1	Hba-a2	-2.18	-0.37
Inositol-trisphosphate 3-kinase A	Itpka	-2.74	-0.90

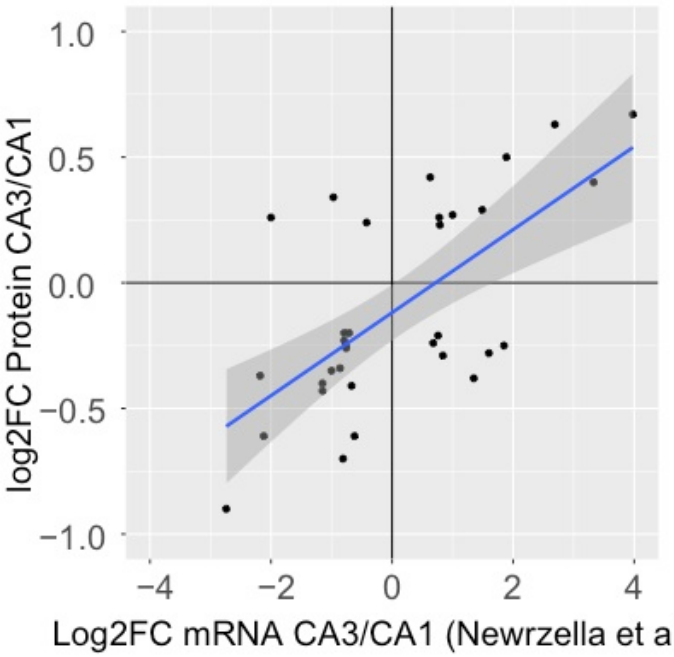


Figure 2-S3: Differences between area CA1 and CA3 proteomes in basal conditions.

Comparison of proteomic data with published mRNA expression data (Newrzella, Pahlavan et al. 2007).
Top: table with all mRNAs/proteins that were significantly different (adj. p-value < 0.05) in expression between area CA1 and area CA3 in Newrzella et al. 2007 and our study. Green: higher expression in area CA3 in both studies. Red: higher expression in area CA1 in both studies. Grey: conflicting results between studies. Numbers expressed as Log2 fold change (Log2FC)
Bottom: Linear correlation between data in Newrzella et al. 2007 and our study (Log2FCProtein = 0.38 *, Log2FCRNA -0.36, p-value of slope = 3.7 e-5 ***, p-value of intercept = ns., R^2 = 0.43)

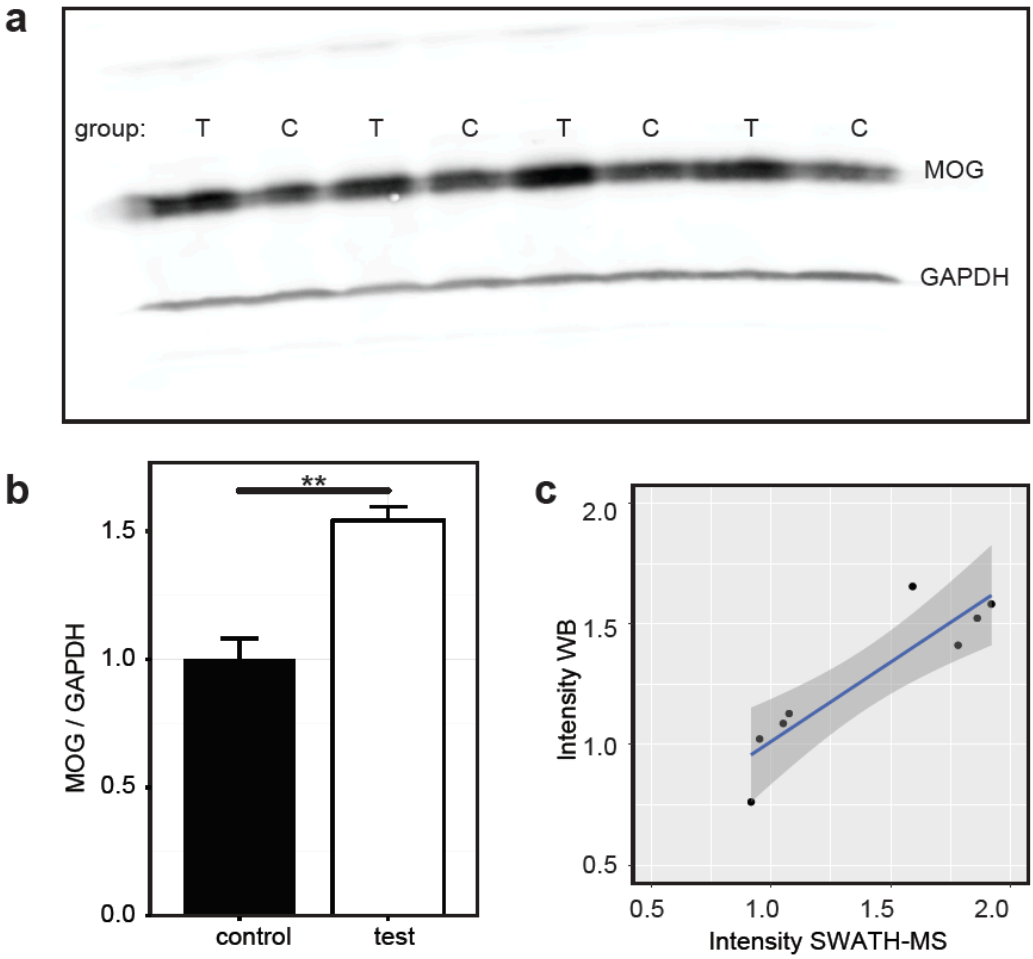


Figure 2-S4: Technical replication with Western blot for myelin oligodendrocyte glycoprotein (MOG) of test vs. control in the 4h time-point following object location recognition in area CA3.

(a) Western blot of MOG using GAPDH as housekeeping protein (T = test, C = control).
(b) Quantitative results of MOG / GAPDH (P = 0.0014 **, error bars = SEM, control n = 4, test n = 4)
(c) Linear correlation between measured SWATH-MS intensity and Western blot values (Intensity WB = 0.66 * intensity MS + 0.35, p-value of slope = 0.002 **, p-value of intercept = ns., R^2 = 0.79)

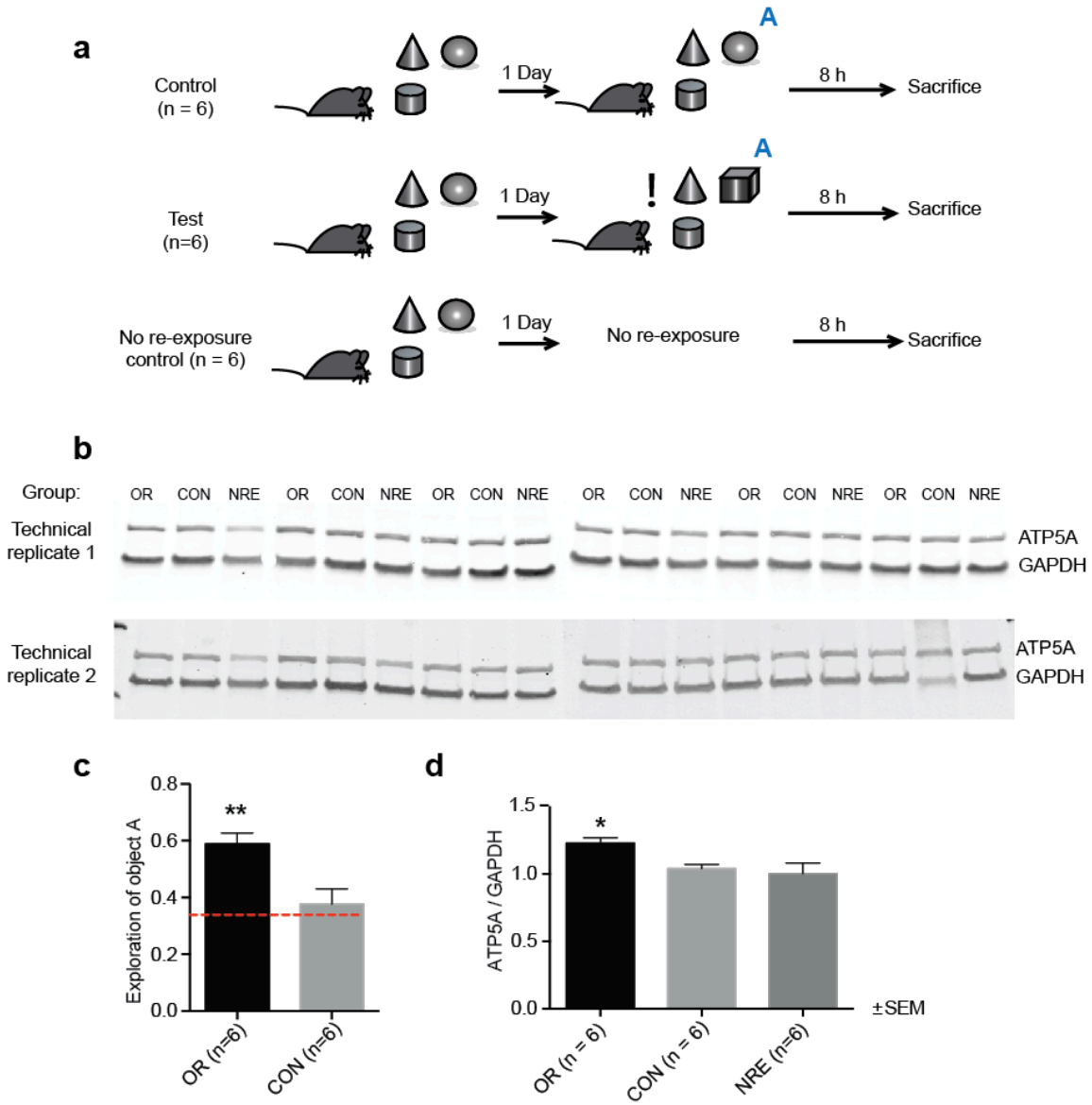


Figure 2-S5: Repetition of the object recognition 8 hour time-point and validation of ATP5A changes

(a) OR exposure and test sessions for control and test animals. A third no re-exposure group was used to determine if the observed effect is specific for object recognition.

(b) Western blots for ATP5A after 8 hours (OR = object recognition, CON = control, NRE = no re-exposure). GAPDH was used as housekeeping control. Two technical replicates were performed.

(c) Exploration of object A (unfamiliar for test animals, familiar for control animals) for test and control animals (** p = 0.0012). The dashed red line indicates chance level exploration of 33%.

(d) Quantitative results for mean of ATP5A/GAPDH ratio from the two replicates. ANOVA p-value * = 0.0253 (post hoc tests: OR – CON adj.p * = 0.03, OR – NRE adj.p * = 0.023)

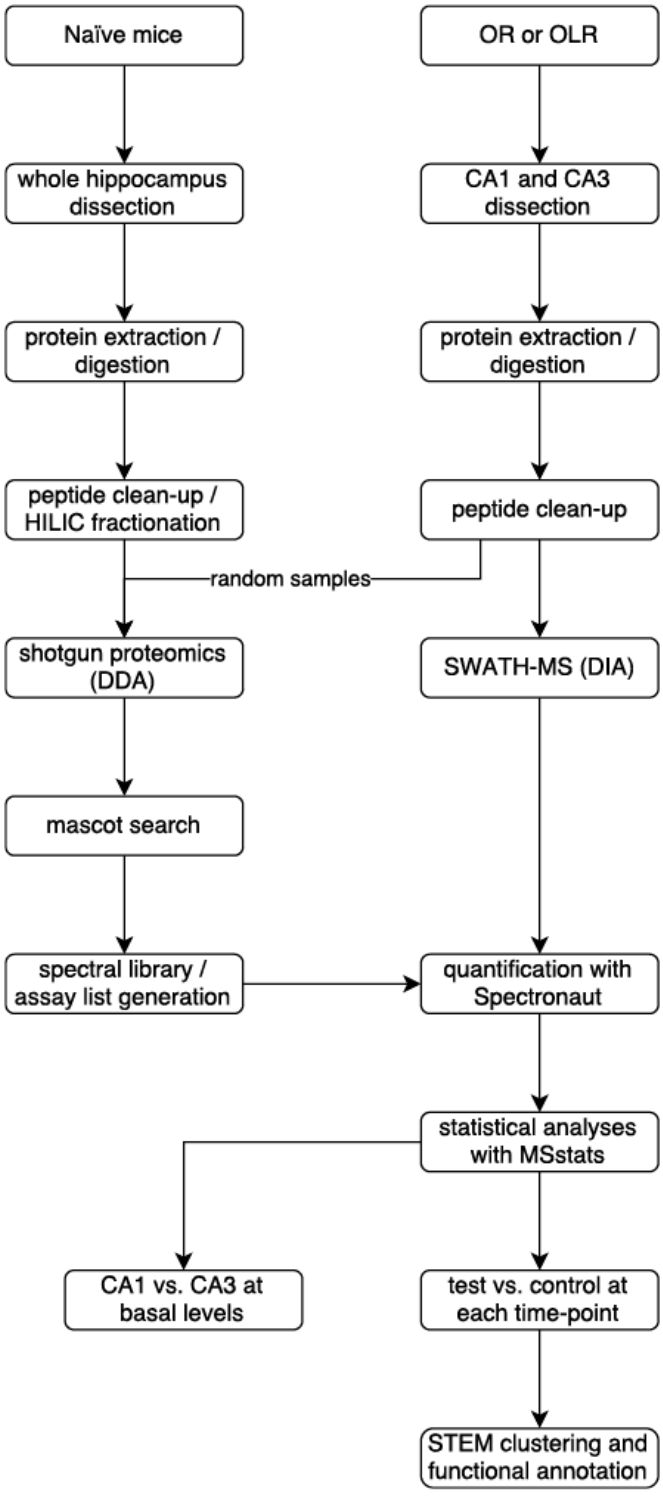


Figure 2S-6: Flowchart of sample processing and data analysis.

Whole hippocampus was dissected from naïve mice and area CA1 and CA3 were dissected following OR and OLR. Proteins were digested and peptides cleaned-up. Peptides from whole hippocampus extracts were further fractionated with HILIC. DDA was performed on fractionated hippocampus samples and random samples from the OR/OLR pool. In parallel SWATH-MS was used for DIA on the OR/OLR samples. DDA data was searched with mascot and a spectral library and Spectronaut assay list generated. Spectronaut was then used for quantification of the DIA data. Statistical analyses were performed with MSstats both for CA1 vs. CA3 under basal condition and test vs. control at each time-point in both area CA1/CA3 and both OR/OLR. STEM clustering and functional annotation was then used to interpret the data

2.9 Supplementary data

These tables and figures appeared in the supplementary data of this publication

See attached CD-ROM

PDF S1: This PDF contains the GO analysis results for proteins significantly higher expressed in area CA1 or in area CA3 at basal conditions.

PDF S2: This PDF contains visual representation of all MSstats results for the OR and OLR paradigms at each time-point and in both sub-regions. Interval plots for log_2FC test/control of each quantified protein (denoted by their uniprot identifiers, one per page) at all time-points are shown. Error bars represent the standard error of the mean.

Table S1: This table contains all MSstats results for the area CA1 vs. area CA3 comparison at baseline. Each row contains the statistical results for a protein denoted by its uniprot identifier.

Table S2: This table contains all MSstats results for the test vs. control comparison in OR and OLR. Each row contains the statistical results for a protein (denoted by its uniprot identifier) at one time-point, in one sub-region and following one paradigm.

Table S3: This table contains all STEM results. Results from OR and OLR and corresponding sub-regions are shown in four separate sheets. A sheet contains all clusters observed in one sub-region following one paradigm. For each cluster the number of assigned proteins, expected proteins and adj. p-value of the cluster are listed. The entry of each cluster is followed by the expression data (log_2FC) of all proteins that were assigned to it at each time-point.

2.10 Scripts used for normalization, group assignment and q-value filtering

```
// SCRIPT 1
// This script C ++ extracts results from a spectronaute fragment report and normalizes them between samples within 15
iRT windows.

using namespace std;
#include<iostream>
#include<fstream>

int main()
{
    // UPDATE THESE TWO const ints before runing
    const int sampleN = 38;             // This has to represent the N of the samples in the experiment
    const int filesize = 5034544;       // This has to represent the size of the specrotnaute file (in lines)
    const int AssayN = filesize / (sampleN * 4);


    string protein[AssayN], peptide[AssayN];
    string runing[30];
    string delete;

    // UPDATE THESE paths before runing
    ifstream input1("PATH/Fragment_Report.xls");            // INPUT FILE for spectronaute fragment report
    ifstream input2("PATH/SWATH_Mouseproteome.txt");        // INPUT FILE for Assay list

    // loads all proteins and peptides from the assay list into protein array and peptide array
    for( int n=0;n<AssayN ;n++){
        input2 >> protein[n];
        input2 >> peptide[n];
    }

    // remove header
    for(int i= 0; i<30; i++){
        input1 >> delete;
    }

    //create normalization matrix, set all values to 0
    double normmat[sampleN][15];
    for(int k = 0; k<sampleN;k++){
        for(int l = 0; l<15;l++){
            normmat[k][l]=0;
        }
    }

    int num=0;
    int check=0;
    double iRT,area;

    //read all iRTs, put them into the correct iRT windows
    for (int j=0; j <filesize; j++){
        for(int i= 0; i<30; i++){
            if(i==13){
                input1 >> iRT;
            }
            else if(i==28){
                input1 >> area;
            }
            else{
                input1 >> delete;
            }
        }

        //Sets maximal area for artifact removal
        if(area>1500){
            area = 1500;
        }

        //iRT intervals
        if(iRT< -20){
            normmat[num][0]=normmat[num][0]+area;
        }
        else if(iRT< -10){
            normmat[num][1]=normmat[num][1]+area;
        }
        else if(iRT< 0){
            normmat[num][2]=normmat[num][2]+area;
        }
        else if(iRT< 10){
            normmat[num][3]=normmat[num][3]+area;
        }
        else if(iRT< 20){
            normmat[num][4]=normmat[num][4]+area;
        }
        else if(iRT< 30){
            normmat[num][5]=normmat[num][5]+area;
        }
        else if(iRT< 40){
            normmat[num][6]=normmat[num][6]+area;
        }
        else if(iRT< 50){
            normmat[num][7]=normmat[num][7]+area;
        }
        else if(iRT< 60){
            normmat[num][8]=normmat[num][8]+area;
        }
        else if(iRT< 70){
```

```
            normmat[num][9]=normmat[num][9]+area;
        }
        else if(iRT< 80){
            normmat[num][10]=normmat[num][10]+area;
        }
        else if(iRT< 90){
            normmat[num][11]=normmat[num][11]+area;
        }
        else if(iRT< 100){
            normmat[num][12]=normmat[num][12]+area;
        }
        else if(iRT< 120){
            normmat[num][13]=normmat[num][13]+area;
        }
        else{
            normmat[num][14]=normmat[num][14]+area;
        }

        area=0;
        // check if new sample is coming
        if(check== (filesize / 4) - 1){
            check = 0;
            num++;
        }
        else{
            check++;
        }

    }

    //create normalization factor matrix
    double normfact[sampleN][15];
    for(int k = 0; k<15;k++){
        for(int l = 0; l < sampleN;l++){
            double average = 0;

            for(int m=0; m < sampleN; m++){
                average = average + normmat[m][k];
            }
            average = average / sampleN;
            normfact[l][k]= average/normmat[l][k];
        }
    }


    input1.close();

    // UPDATE THESE paths before runing
    ifstream input3("PATH/Fragment_Report.xls");
    ofstream output1("PATH/Experiment_Normalized.txt");

    // export peptides of interest with adjusted normalization matrix
    output1 << "protein" << "\t" << "peptide" << "\t" << "filename" << "\t"<< "Fragtype" << "\t"<< "num" << "\t"<<
"PrecCharge" << "\t"<< "Fragcharge" << "\t" << "Qvalue" << "\t"<< "iRTemp" << "\t"<< "iRTpred" << "\t" << "peakarea" <<
"\t"<< "normalizedpeakarea"<< endl;

    for(int i= 0; i<30; i++){
        input3 >> delete;
    }
    num=0;
    check=0;
    int window;



    for (int j=0; j <filesize; j++){

        // read input
        for(int i= 0; i<30; i++){
            if(i==13){
                input3 >> iRT;
            }
            else if(i==28){
                input3 >> area;
            }
            else{
                input3 >> runing[i];
            }
        }

        //check iRT window
        if(iRT< -20){
            window=0;
        }
        else if(iRT< -10){
            window=1;
        }
        else if(iRT< 0){
            window=2;
        }
        else if(iRT< 10){
            window=3;
        }
        else if(iRT< 20){
            window=4;
        }
        else if(iRT< 30){
            window=5;
        }
```

```
        else if(iRT< 40){
            window=6;
        }
        else if(iRT< 50){
            window=7;
        }
        else if(iRT< 60){
            window=8;
        }
        else if(iRT< 70){
            window=9;
        }
        else if(iRT< 80){
            window=10;
        }
        else if(iRT< 90){
            window=11;
        }
        else if(iRT< 100){
            window=12;
        }
        else if(iRT< 120){
            window=13;
        }
        else{
            window=14;
        }


        for(int m=0;m<AssayN;m++){
            if(runing[10]==peptide[m] && runing[9]==protein[m]){


                output1 << protein[m] << "\t" << peptide[m] << "\t" << runing[1] << "\t" << runing[25] << "\t" <<
runing[26]<< "\t" << runing[17] << "\t" << runing[24] << "\t" << runing[6] << "\t"<< runing[4] <<"\t"<< iRT <<"\t"<< area
<<"\t"<<area * normfact[num][window]<< endl;
                m=AssayN;
            }
        }
        if(check== (filesize / 4) - 1){
            check = 0;
            num++;
        }
        else{
            check++;
        }
    }

    return 0;
}

// SCRIPT 2
// This C++ script takes the output file from SCRIPT 1 and removes duplicates entry in that might still be present in the
result file

using namespace std;
#include<iostream>
#include<fstream>

int main()
{
    // UPDATE THESE TOW const ints before runing
    const int filesize = 5034544;   // This has to represent the size of the specrotnaute file (in lines)
    const int sampleN = 38;         // This has to represent the N of the samples in the experiment
    const int AssayN = filesize / (sampleN * 4);

    string protein[AssayN], peptide[AssayN],precursor[AssayN];
    int sanity[AssayN];
    int expandedsanity[AssayN * 4];
    string runing[12];
    string delete;

    // UPDATE THIS path for the input file before running
    ifstream input1("PATH/CA3_export_spec6_iRTnorm_proteome_total.txt");
    for(int j = 0;j<12;j++){
        input1 >> delete;
    }

    for(int i =0;i<AssayN;i++){
        for(int j = 0;j<12;j++){
            input1 >> delete;
        }
        for(int j = 0;j<12;j++){
            input1 >> delete;
        }
        for(int j = 0;j<12;j++){
            input1 >> delete;
        }
        input1 >> protein[i];
        input1 >> peptide[i];
        input1 >> delete;
        input1 >> delete;
        input1 >> delete;
        input1 >> precursor[i];
        input1 >> delete;
        input1 >> delete;
        input1 >> delete;
        input1 >> delete;
        input1 >> delete;
```

```
        input1 >> delete;
        sanity[i]=1;
    }

    for(int i =0;i <AssayN;i++){
        for(int j=i+1;j <AssayN;j++){
            if(peptide[i]==peptide[j]&&protein[i]==protein[j]&&precursor[i]==precursor[j]){
                sanity[i]=0;
                sanity[j]=0;
            }
        }
    }

    input1.close();

    // UPDATE THESE paths for the input and output file before running
    ifstream input2("PATH/Experiment_Normalized.txt");
    ofstream output1("PATH/Experiment_Normalized_NoDuplicates.txt");
    for(int j = 0;j<11;j++){
        input2 >> delete;
        output1 << delete <<  "\t";
    }
    input2 >> delete;
    output1 << delete << endl;


    int check =0;
    for(int i =0; i<filesize;i++){
        for(int j = 0;j<12;j++){
            input2 >> runing[j];
        }
        if(expandedsanity[check]==1){
            for(int j = 0;j<11;j++){
                output1 << runing[j] << "\t";
            }
            output1 <<runing[11]<< endl;
        }

        if(check== (AssayN * 4) - 1){
            check =0;
        }
        else{
            check++;
        }
    }


    return 0;
}

// SCRIPT 3
// This C++ Scripts takes the output from Script2 and assings groups and conditinos as denoted in file grouping.txt. It
returns a tab delimited file of the results with correct groups and conditions

using namespace std;
#include<iostream>
#include<fstream>


int main()
{

    // UPDATE THESE TOW const ints before runing
    const int filesize = 5010224;   // This has to represent the size of the specrotnaute file (in lines)
    const int sampleN = 38;         // This has to represent the N of the samples in the experiment
    const int AssayN = filesize / (sampleN * 4);

    // UPDATE THESE Paths for input / output
    ifstream input1("PATH/grouping.txt");
    ifstream input2("PATH/Experiment_Normalized_NoDuplicates.txt");
    ofstream output1("PATH/Experiment_Normalized_NoDuplicates_GroupsAssigned.txt");
    string condition[sampleN],replicate[sampleN],run[sampleN],runing[12];
    input1 >> runing[1];
    input1 >> runing[1];
    input1 >> runing[1];
    for(int i=0;i<sampleN;i++){
        input1 >> condition[i];
        input1 >> replicate[i];
        input1 >> run[i];
    }

    for(int i=0;i <12; i++){
        input2 >> runing[i];
        output1 << runing[i]<< "\t";
    }
    output1<<"condition"<< "\t"<<"replicate"<< "\t"<<"run" << endl;

    int check=0;
    int num=0;

    for(int j=0; j<filesize;j++){
        for(int i=0;i <12; i++){
            input2 >> runing[i];
            output1 << runing[i]<< "\t";

        }

        check++;
```

```
        output1 << condition[num]<< "\t" << replicate[num]<< "\t"<<run[num] << endl;

        if(check> (AssayN * 4) - 1){
            check =0;
            num++;
        }



    }

    return 0;
}

// SCRIPT 4
// This C++ script takes the output from SCRIPT 3 and performs a qvalue filtering. Transitions with a q > 0.01 in more
than 25% of all samples are removed for all samples, Transitions with a q > 0.01 in less than 25% of all samples are
removed for the respective sample only

using namespace std;
#include<iostream>
#include<fstream>


int main()
{

    // UPDATE THESE TOW const ints before runing
    const int filesize = 5010224;   // This has to represent the size of the specrotnaute file (in lines)
    const int sampleN = 38;         // This has to represent the N of the samples in the experiment
    const int AssayN = filesize / (sampleN * 4);

    ifstream input1("PATH/Experiment_Normalized_NoDuplicates_GroupsAssigned.txt");

    string runing[16];
    string delete;
       string proteinlist[AssayN * 4];
    int sanity[AssayN * 4];
    for(int j=0; j<AssayN * 4;j++){
        sanity[j]=0;
    }
    double qvalue;

    for(int i=0;i <15; i++){
        input1 >> delete;
    }

    int stop =0;
    int check=0;
    for(int j=0; j<filesize;j++){

        for(int i=0; i<7; i++){
            input1 >> runing[i];
        }
        if(stop == 0){
            proteinlist[j] = runing[0];
        }
        input1 >> qvalue;
        for(int i=8;i <15; i++){
            input1 >> runing[i];
        }

        if(qvalue<0.01){
            sanity[check]=sanity[check]+1;
        }
        if(check==AssayN * 4 - 1){
            check=0;
            stop=1;
        }
        else{
            check++;
        }
    }
    int proteinsanity[AssayN * 4];
    for(int i=0;i<AssayN * 4;i++){
        proteinsanity[i] =0;
        for(int j=0;j<AssayN * 4;j++){
            if(sanity[j]> (sampleN * 3 / 4) && proteinlist[i]==proteinlist[j]){
                proteinsanity[i]++;
            }
        }
    }



    ifstream input2("PATH/Experiment_Normalized_NoDuplicates_GroupsAssigned.txt");
    ofstream output1("PATH/Experiment_MSStatsInputFile.txt");
    output1 <<"ProteinName"<< "\t" <<"PeptideSequence"<< "\t" <<"PrecursorCharge"<< "\t" <<"FragmentIon"<< "\t"
<<"ProductCharge"<< "\t" <<"IsotopeLabelType"<< "\t" <<"Condition"<< "\t" <<"BioReplicate"<< "\t" <<"Run"<< "\t"
<<"Intensity" << endl;

    for(int i=0;i <15; i++){
        input2 >> delete;
    }
    check=0;
    for(int j=0; j<filesize;j++){

        for(int i=0; i<7; i++){
            input2 >> runing[i];
```

```
        }

        input2 >> qvalue;
        for(int i=8;i <15; i++){
            input2 >> runing[i];
        }

        if(proteinsanity[check] > 4 && sanity[check] > (sampleN * 3 / 4) && qvalue<0.01){
            output1 << runing[0] << "\t"<< runing[1] << "_"<<runing[0] << "\t"<< runing[5] << "\t"<< runing[3]<<runing[4]
<< "\t"<< runing[6] << "\t"<< "L" << "\t"<< runing[12] << "\t"<< runing[13] << "\t"<< runing[14] << "\t"<< runing[11]
<<endl;
        }

        if(check==AssayN * 4 - 1){
            check=0;
        }
        else{
            check++;
        }
    }

    return 0;
}
```

3 Conclusions and Outlook

This thesis provides evidence that the proteome of area CA1 and CA3 show distinct differences at basal conditions. It further shows, that two different memory paradigms, object recognition (OR) or object location recognition (OLR), both induce changes in protein expression at multiple time-points following the test phase. The observed changes differ between area CA1 and area CA3. In area CA1 both learning paradigms induce similar changes, whereas the changes observed in area CA3 are more specific. In section 2.4 we explored how our findings integrate in the current literature and how recognition of novelty and memory could be interdependent through ETC dependent modulation of neuron excitability. In the following section we provide a more in depth conclusion about strengths and limitations of this thesis, what further questions would have to be answered to provide a causal link between ETC proteins and memory and how this could have implications in pathologies where ETC dysfunctions are observed.

3.1 Differences between area CA1 and CA3 at basal levels

The differences between protein expression in area CA1 and CA3 under basal conditions have not yet been described in the literature and will be of interest for researchers in this field. In combination with published mRNA data (Newrzella, Pahlavan et al. 2007) we were able to identify genes with robust expression difference between the area CA1 and area CA3 both on the mRNA and protein level that could act as markers for these sub-regions. Additionally, researchers interested in the importance of selected proteins for memory can refine their hypotheses based on the known functions of area CA1 and CA3 from the literature and the expression data from this thesis.

The fact that there are such extensive differences in protein expression (30% of all quantifiable proteins) between two similar sub-regions illustrates the importance of sub-region or even cell type specific analyses. Researchers often use whole hippocampal tissue to study molecular components important for learning and memory, but the results have to be interpreted with caution. While consistent memory or learning dependent hippocampus wide effects of selected molecular components are strong arguments for their importance in these processes,

absence of such observations does not indicate the opposite. It is conceivable that many molecular components that are of importance for memory formation are specific for sub-regions and type of memory, making it impossible to study their function with hippocampus wide analyses. Therefore, transcriptional and proteomic studies of more and more refined sub-divisions of classically studied brain structures could open the gates for intriguing new findings.

While this thesis analyzed differences between whole area CA1 and CA3 dissections, further separating the dorsal and ventral parts of both area CA1 and CA3 could achieve even better specificity. Dissections could be further refined to allow a distinction between areas CA1, CA2 and CA3. Area CA2 is a much smaller sub-region that is located between area CA1 and CA3, which has been shown to have its own unique properties and functions (Dudek, Alexander et al. 2016). Due to its small size and the difficulty to precisely dissect it from area CA1 and CA3, CA2 is often ignored. Precise dissection of area CA2 would require slicing the hippocampus orthogonal to the dorsal-ventral axis multiple times and dissecting area CA2 from individual slices. Whole hippocampus area CA1 and CA3 extracts from a single animal contain enough protein to perform ~30-50 SWATH-MS analyses each assuming a minimum requirement of 20µg for the sample preparation, so further sub-division of dissections would be feasible, though this would increase the time requirement for individual dissections, and complicate the overall experimental design.

3.2 Relevance of behavioral paradigms used in this thesis

Only a hand full of studies has examined the effect of memory formation on the proteome of the hippocampus (Monopoli, Raghnaill et al. 2011, Rao-Ruiz, Carney et al. 2015, Borovok, Nesher et al. 2016) and this thesis is the first to look at changes in area CA1 and CA3 specifically. One of the strengths of this thesis is the selection of relevant learning paradigms to study hippocampus dependent memory and the appropriate control groups that allow controlling for effects that are independent of memory formation.

OR and OLR have been shown to depend mainly on hippocampal function (Cohen and Stackman 2015). While some studies have argued that OR is independent of hippocampal function (Lee, Hunsaker et al. 2005), these studies relied on multiple

exposures to the same objects, a process which induces familiarity. Familiarity in contrast to recall is a semantic form of memory, is independent of recollection of details associated with the objects and does not require hippocampal functions (Yonelinas, Kroll et al. 2002). For single exposure, as used in this thesis, OR has been shown to rely on the hippocampus (Broadbent, Gaskin et al. 2010). In addition to their dependency on the hippocampus, both paradigms model novelty recognition and memory formation due to intrinsic motivation (curiosity) in contrast to tasks that use external motivation such as food reward. This eliminates potential effects from reward prediction or reward which are mediated by dopaminergic pathways that originate in the VTA and innervate the hippocampus (Martig and Mizumori 2011). Alternative memory paradigms often used in the field, such as contextual fear conditioning, rely on strong but artificial learning stimuli, and while depending on hippocampal function they are also mediated by other brain regions, i.e. the amygdala in the case of contextual fear conditioning (Kochli, Thompson et al. 2015).

The use of multiple control groups in this thesis, one at each time-point, allows controlling for circadian effects (around 700 genes have been shown to fluctuate in expression over the course of a day in the human hippocampus on the mRNA level (Li, Bunney et al. 2013)) and for activity and exploration induced effects. This is often overlooked in comparable studies, where expression changes due to memory are obtained by comparing test animals from time-points to either naïve cage controls or to controls directly sacrificed after the tasks at a different time of the day, making it impossible to distinguish between circadian, activity induced and memory related changes.

To recapitulate, in this thesis we use hippocampus dependent recognition paradigms that conform to natural exploration, novelty recognition and memory formation in mice and use control groups that allow controlling for both circadian and activity dependent effects. This allows for a read-out that is very specific to the recognition paradigms and hippocampal sub-regions.

3.3 The source of the observed expression changes

The sources of the observed changes are differences in experience between control and test animals. While often referred to as tasks for memory, it has to be

noted that both object recognition and object location recognition encompass other sources that could induce protein expression changes independent of the formation of new memory.

In the test group there are two potential sources that could induce the observed changes. The first is recognition of novelty as experienced in both OR and OLR, which is specific for test animals and is absent in control animals. It has been shown that novelty recognition is a process that depends on the hippocampus (Grunwald and Kurthen 2006) and increases activity in hippocampus area CA1 (Larkin, Lykken et al. 2014). Thus, recognition of novelty itself could be responsible for some expression changes, though we hypothesize in section 2.4 that novelty recognition and subsequent memory formation are interdependent.

The second source is the exploration of the altered context and subsequent memory formation, which is also specific for test animals and absent in control animals. It is established that episodic memory formation requires *de novo* protein synthesis in the hippocampus (Morris, Inglis et al. 2006), so it is expected that some of the observed protein expression changes depend on this process.

While we assume that the majority of observed expression changes are induced by these two sources in test animals, there is a third potential source, which comes from the control animals. It could be argued that memory consolidation as experienced by control animals but not by test animals could be responsible for some of the observed expression changes. Previous studies have demonstrated though that exploration of familiar environments does not increase hippocampal activity, as shown with early gene imaging of c-Fos (VanElzakker, Fevurly et al. 2008) and does not activate CREB (Winograd and Viola 2004), a transcription factor important for memory. Therefore extensive expression changes contributed to re-exploration of a familiar context are not expected. For both OR and OLR it is not possible to design a single control group that can control for both activity and exploration and does not induce potential memory consolidation. For the validation of ETC protein changes 8 hours following OR we have included a second control group without re-exploration to monitor for this eventuality and demonstrated that the effect is specific for the test group in this case.

In summary, there is not a single source that can be attributed for all observed expression changes with certainty. We argue that the major part of proteomic

changes is attributed to novelty recognition and subsequent memory formation in test animals and validated this for selected proteins.

3.4 Data interpretation with clustering analyses

While studying a complex system such as the hippocampus during natural memory tasks provides results with high relevance for the field of research, the observed changes in this study were considerably lower than comparable changes in proteomic studies that analyze systems after stronger perturbations, such as altered metabolic steady states (Costenoble, Picotti et al. 2011). This makes reliable reproduction of effects observed for single proteins challenging and the change of individual proteins should be interpreted with caution.

In order to achieve a better understanding of system wide changes post hoc approaches can be used to perform analyses over the whole dataset. While single proteins with low fold change don't convey a strong message, observing multiple proteins with consistent changes increases confidence in an underlying biological cause. In this case statistical clustering was used to identify expression patterns that are enriched over the whole time-series.

This poses the question if such an analysis should be performed on the whole data set or a sub-set (i.e. only significant values). Performing it on significant values only requires to either restricting it to proteins that are significant at all time-points, or setting fold changes of non-significant entries to 0. Restricting it to proteins with significant differences at all time-points reduces the list to only a hand full, insufficient for clustering analyses. Setting non-significant time-points to 0 leads to other problems, since it induces many type II errors, where null-hypotheses are accepted, even though the alternative hypothesis is true and the fold-changes are wrongfully set to 0. Using an adjusted p-value cutoff will result in a large number of type II errors.

On the other hand, the algorithm that was used for statistical clustering predicts the number of proteins that will follow a given expression profile due to type I errors by random chance within the data-set, and indicate the false discoveries for each profile (Ernst, Nau et al. 2005, Ernst and Bar-Joseph 2006).

Therefore mean values for $\log_2$ fold-changes between test and controls at each time-point were used for statistical clustering without any significance cutoff similar

to previously published studies that used the same approach (Han, Yang et al. 2008, Ernst and Kellis 2010, Ghandhi, Sinha et al. 2011, Ho, Papp et al. 2013, Lu, Kim et al. 2014, Razy-Krajka, Lam et al. 2014) including proteomic studies (Scholz, Svensson et al. 2008). For each profile we indicate the expected false discoveries due to type I errors. In order to test the algorithm for effects that could arise due to artifacts in the data, the same analysis was performed with a randomized data-set. In this data-set all fold-changes within the experiment were re-assigned randomly to other proteins and time-points. With the randomized data-set no significant clusters were found by the algorithm.

In summary, we used statistical clustering to interpret the whole data set. We opted for an approach that uses raw fold change values without any significance cutoff and ensured that the findings are not originating from data artifacts with a randomized data-set.

3.5 Cellular specificity

A limit of this thesis is the specificity of the data. While proteomic data of area CA1 and CA3 during recognition tasks is a novelty in the field and adds additional complexity to the understanding of molecular processed during memory formation in the hippocampus, it is constricted to whole tissue extracts and no further information about changes in specific cell populations can be gained. Volume wise both area CA1 and CA3 comprises about 50% neurons and 50% glial cells. Therefore, it can't be determined if the observed changes appear in all cells, or only specific sub-populations, such as excitatory neurons for example, which would dampen observed changes. Additionally, it has been shown that exploration of a novel environment only activates up to ~40% of all neurons both in area CA1 and CA3 (Guzowski, McNaughton et al. 1999), which would further dampen observed changes in activated neurons. While a protein with equal expression in neurons and glial cells might be observed as having a low fold change between conditions in the whole tissue extract, the change could be much stronger in the activated neuronal population.

Addressing this question would require the separation of neurons from glial cells or even better activated neurons from non-activated neurons. On prominent approach is fluorescence activated cell sorting (FACS). With this technique,

fluorophore-conjugated antibodies against proteins that are specific for one sub-set of cells are used to mark them. A flow cytometer is then used, to separate marked from unmarked cells. While this approach is relatively straightforward in cell cultures where cells are loosely connected to each other, it is more difficult in tissue samples. In tissue samples, cells have a strong adhesion to neighboring cells. To separate them, the extracellular matrix has to be digested in order to loosen the adhesion and then the tissue has to be dissociated mechanically, usually by using a narrow glass pipette.

The problem with brain tissue is that neurons have a very complex morphology with extensive axonal and dendritic arborization. While dissociation of hippocampal tissue in adult rats is possible (Guez-Barber, Fanous et al. 2012), in the process most of the axons and dendrites are ripped of the cell bodies, the later of which can then be sorted. Many of the proteins of interest are located in the axons and dendrites and changes in them would not be observable with this approach. While the initial proposal of this thesis considered using FACS followed by SWATH-MS for cell-specific proteomic read-outs, preliminary experiments demonstrated a high amount of debris and low amounts of recoverable proteins, insufficient for mass-spectrometry.

The necessity of using whole tissue extracts for mass spectrometry has implications on the findings of altered ETC proteins in this thesis. It cannot be concluded if these changes happen uniformly in the tissue, in neurons or glial cells exclusively, or in an even smaller sub-set of neurons or glial cells, i.e. only activated neurons. Neurons rely heavily on mitochondria in order to maintain homeostasis and they use up to 96% of all ATP produced in gray matter (Zhu, Qiao et al. 2012) so we assume that most of the observed changes represent alterations in neurons or activated neurons, but further experiments are necessary to conclusively demonstrate this.

A potential approach would be immunohistological staining of ETC proteins in hippocampal slices following object recognition or object location recognition, which would not only answer the question in what cells mitochondrial ETC compositions are changed, but also where in these cells the changes occur, i.e. if they are exclusive for axons or dendrites, both known for high mitochondria density and activity dependent mitochondria transport (MacAskill, Brickley et al. 2009, Schwarz 2013).

3.6 ETC proteins and memory

In the discussion of section 2.4 we have hypothesized that dynamic ETC protein changes could be important for episodic memory by modulating excitability of CA1 pyramidal neurons.

The observation, that ETC protein change in expression following recognition paradigms is not sufficient for a causal link between ETC proteins and memory. In order to establish such a link further experiments are necessary.

First, the dependency of memory on dynamic ETC changes has to be demonstrated. For this ETC reduction or increase would have to be inhibited or stimulated and altered memory demonstrated. Since ETC proteins are crucial for homeostasis and complete ETC inhibition is lethal for cells (Birsoy, Wang et al. 2015), specific and conditional inhibition would be the only viable approach. Inhibiting or activating all ETC components directly would proof difficult, so a better approach would be to inhibit or activate upstream regulators of ETC proteins. There are multiple potential regulators that have been described in the literature, so identifying which of them is mediating ETC changes in OR and OLR would be the first step.

Regulators for ETC proteins that have been described in the literature can be sub-divided into two broader classes, transcriptional regulators and post-transcriptional regulators. Quantification of ETC mRNA following OR and OLR could determine which class is likely acting as upstream regulator. If changes on the mRNA level reflect changes on the protein level we would expect a transcriptional regulator, if not a post-transcriptional regulator is more likely.

If there is a transcriptional basis to the changes, transcription factors that regulate ETC genes are good candidates. The majority of the ETC components are encoded in the nuclear DNA, but some of them are encoded in the mitochondrial DNA (mtDNA). In this thesis only ETC components that are encoded in the nuclear DNA could be quantified, so alternative quantitative approaches (western blot or targeted proteomics) would be necessary to first determine if the observed effects are specific for nuclear encoded ETC components or not.

If changes are not specific for nuclear encoded ETC components, a potential transcriptional regulator of ETC proteins is *Tfam* (mitochondrial transcription factor A), which has been shown to mediate calcium-dependent increase of both nuclear

and mitochondrial ETC genes both on the mRNA and protein level (Au, Yeh et al. 2005). *Tfam* itself can be actively regulated by PKA (cAMP dependent protein kinase) mediated phosphorylation, which marks it for degradation (Lu, Lee et al. 2013). Interestingly, chronically increased PKA activity has been linked to disrupted recognition memory and spatial memory (Giralt, Saavedra et al. 2011), but it has to be noted that PKA regulates many other genes than *Tfam*, some of which have been directly linked to memory (Park, Havekes et al. 2014). This would make PKA a bad target to study *Tfam* and its potential downstream effects on memory through ETC proteins. In contrast, conditional overexpression, knockout or knockdown (Ekstrand and Larsson 2002) of *Tfam* during or following memory tasks could yield a causal link between ETC protein expression and memory.

If the observed effects are specific for nuclear encoded ETC components, nuclear respiratory factor 1 and 2 (*Nrf1* and *Nrf2*) (Scarpulla 1997, Johar, Priya et al. 2012) or their upstream regulator Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α or *Ppargc1a*) would be better candidates, since they are nucleus specific. Activity-dependent transcriptional regulation of *Nrf1* has been

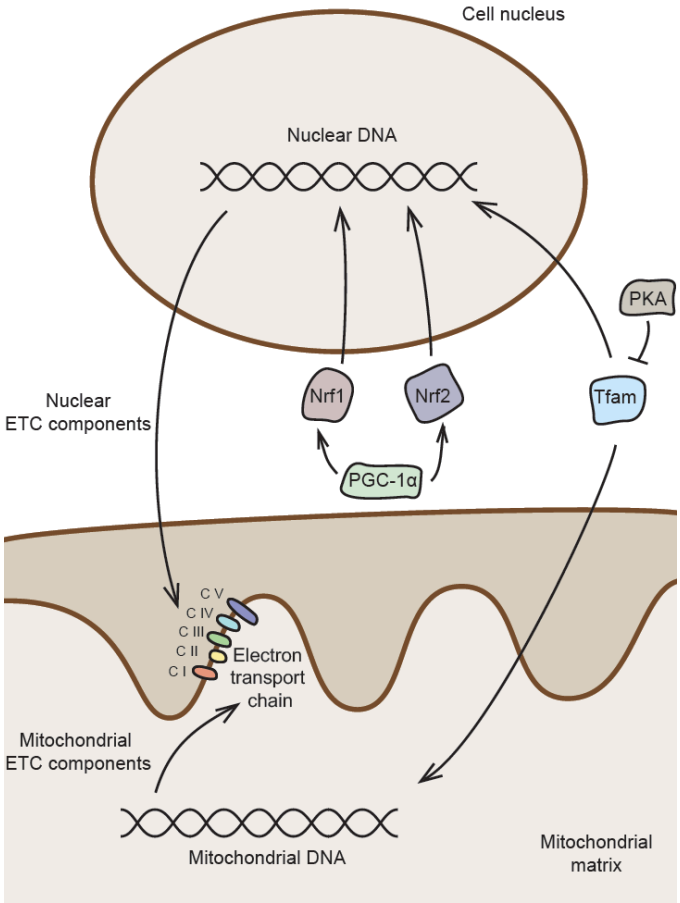


Figure 3-1: Potential transcriptional regulators of ETC components. Transcription could be regulated by *Tfam* required for nuclear and mitochondrial encoded ETC components and itself regulated by PKA, or by Nrf1/Nrf2 which are specific for nuclear encoded ETC components and regulated by PGC-1α

demonstrated in cultured rat visual cortical neurons (Yang, Liang et al. 2006) and overexpression of *Nrf2* in the hippocampus of mice of an Alzheimer's disease model improved spatial learning (Kanninen, Heikkinen et al. 2009).

If ETC protein expression changes are not accompanied by similar changes on the transcriptional level, translational or post-translational mechanisms for ETC are potential up-stream regulators of ETC proteins. A regulator for targeted translation of ETC mRNAs has been identified with the ribonucleoprotein Y-box binding protein 1 (*Ybx1*). *Ybx1* has been shown to bind to multiple ETC mRNAs in HeLa cells and move them from polysomes, complexes of mRNA with ribosomes that are actively translated, into mRNPs, mRNA which are bound to protein complexes and which are translationally silent (Matsumoto, Uchiumi et al. 2012). Knockdown of *Ybx1* leads to up-regulation of mitochondrial function through increased ETC protein translation, and concordantly overexpression of *Ybx1* suppresses translation of ETC proteins. An alternative mechanism for post-translational regulation is degradation of ETC proteins through the ubiquitin-proteasome system

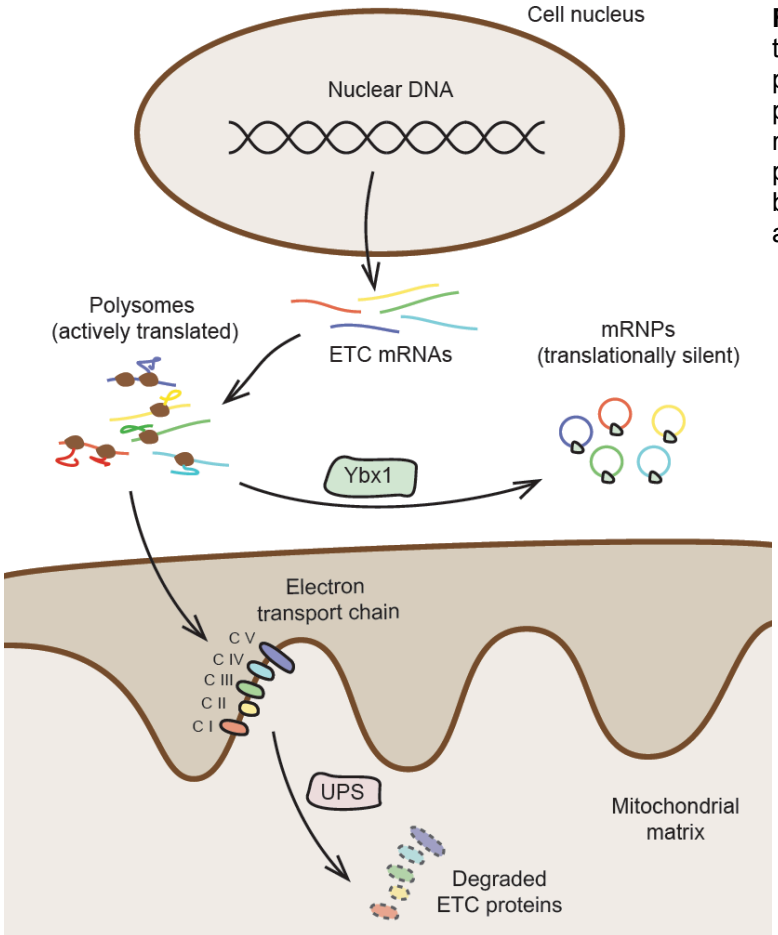


Figure 3-2: Potential post-transcriptional regulators of ETC proteins. Regulation of ETC protein translation could be mediated by *Ybx1* dependent polysome to mRNP alteration or by UPS mediated degradation of assembled ETC proteins.

(UPS), shown to control multiple aspects of mitochondrial functions (Franz, Kevei et al. 2015). It can selectively tag proteins by conjugation with ubiquitin followed by degradation (Nandi, Tahiliani et al. 2006). Many inner mitochondrial proteins can be conjugated to ubiquitin and ubiquitin system components localize to mitochondria (Lehmann, Udasin et al. 2016), allowing the UPS to potentially function within mitochondria.

To recapitulate, if dynamic ETC protein expression changes are important for episodic memory, underlying transcriptional regulators could mediate their increase and decrease as observed in OR and OLR. Potential candidates are *Tfam*, *Nrf1*, *Nrf2* and *Ppargc1a*, depending on whether the effects are specific for nuclear encoded ETC components or not. Alternatively, post-transcriptional mechanisms could mediate the observed effects. In this case, potential candidates are *Ybx1* for translation control or the UPS for targeted degradation.

To establish a causal link between dynamic ETC changes and memory conditional overexpression, knockdown or knockout of these candidate regulators in mice in combination with memory tasks would be needed.

3.7 ETC proteins and pathologies

Providing a causal link between ETC protein expression change, their up-stream regulators and memory could have important implications for multiple pathologies. Mitochondrial dysfunctions have been observed in aging (Navarro, Lopez-Cepero et al. 2008, Navarro and Boveris 2010) and in other pathologies including Alzheimer's disease (AD) (Maruszak and Zekanowski 2011). How mitochondrial dysfunctions contribute to these pathologies is debated.

Classical hypotheses argue that interactions between mitochondrial dysfunction, radical oxygen species (ROS) and neurodegeneration contribute to the pathologies. In aging, an established hypothesis for age related cognitive decline is the free radical theory (Harman 1956). This theory proposes that mitochondrial produced radical oxygen species (ROS) leads to oxidative damage of mitochondria. This then leads to stronger mitochondrial dysfunctions and a subsequent increased ROS production rate. If the oxidative damage reaches an extensive level, cells undergo apoptosis, which then leads to neurodegeneration. Therefore, ROS are argued to be both cause and effect of age related cognitive

decline through a vicious cycle of increased oxidative damage, increased mitochondrial dysfunction and neurondegeneration.

In AD models similar mitochondrial dysfunctions are observed prior to Aβ amyloid aggregation (Moreira, Carvalho et al. 2010), which supports a hypothesis similar to the free radical theory for normal aging, where mitochondrial dysfunctions and resulting oxidative damage contribute to neurodegeneration and the resulting memory impairment independent of Aβ amyloid aggregates.

Both These hypotheses rely on the same underlying mechanisms as causes for cognitive dysfunctions, where increased oxidative damage leads to neurodegeneration. There are alternative hypotheses that challenge this view. They argue that mitochondrial dysfunctions are the cause of cognitive impairments, but in a process that is independent of oxidative damage and independent of neurodegeneration.

Naturally occurring mutations during mtDNA replication have been proposed as alternative cause of age related mitochondrial dysfunctions (Payne and Chinnery 2015). Animal models with increased mutation rates of mtDNA show accelerated aging phenotypes with no indication of increased oxidative damage (Trifunovic, Wredenberg et al. 2004, Kujoth, Hiona et al. 2005). It has been shown that age related dysfunctions are accompanied by decreased enzyme function of ETC complexes (Sandhu and Kaur 2003), which could be a cause of the impairments.

Additionally, recent studies have demonstrated that AD phenotypes can be alleviated by amyloid removal in mice (Sevigny, Chiao et al. 2016). While this does not completely rule out a contribution of oxidative damage to neurodegeneration in AD models it rules out oxidative damage as the primary cause of AD. While neurodegeneration has been proposed as the main cause of cognitive impairment in AD patients, cognitive fluctuations observed in patients support contributions of other variable causes (Escandon, Al-Hammadi et al. 2010). One proposed cause for cognitive impairments are dysfunctions in intrinsic neuronal excitability and neuronal networks (Santos, Pierrot et al. 2010).

To conclude, if ETC proteins have an important function in memory, cognitive impairments in aging and AD could be a cause of dysfunctions in ETC mediated neuronal excitability. This could be contributed to either an overall decrease of ETC function, or impairments in the dynamic system through which ETC proteins are temporally regulated. In this case, novel therapeutic approaches that aim at

restoring normal ETC function and dynamic ETC modulation could be developed to combat cognitive decline experienced in aging and AD.

Appendix A

A simple and fast method for tissue cryohomogenization enabling multifarious molecular extraction

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Abstract

Molecular research in neuroscience often demands parallel analysis of DNA, RNA, protein and/or other molecules from a given brain region. However the quality and quantity for two or more distinct desired products is typically compromised if extracted from a single biological sample. One solution is to use multiple animals, and in doing so obtain near-identical samples that can each be reserved for a given molecular class, but this approach is clearly non-ideal from both an operational and ethical perspective. Thus, we refined the methods for cryohomogenization prior to molecular extraction such that a single experimental sample can be easily divided into aliquots and either processed immediately or stored until needed. Using western blot, nanodrop UV/V spectrometry, and a bioanalyzer, we show that cryohomogenized hippocampus samples provide high-quality RNA and protein without significant loss in abundance. The method may be particularly advantageous for parallel molecular extraction from structures with known hemispheric lateralization, such as the hippocampus, parietal cortex, suprachiasmatic nucleus, and amygdala.

Introduction

Many molecular experiments in the neurosciences require parallel isolation of multiple products from a given tissue or cell population. While this is easily doable for homogeneous samples that can be evenly divided prior to molecular extraction, parallel analysis becomes problematic for heterogeneous tissue because methods for the efficient isolation of various molecular classes are often incompatible. This conundrum is especially relevant for molecular neuroscience, since the brain contains many highly heterogeneous structures, and obtaining independent samples therefore typically requires sacrificing independent animals. However, given the expense of animal research, the time required to obtain specific genotypes and repeat experiments, the limitation of specific experimental consumables, and a policy from animal care councils to reduce experimental animal numbers (Flecknell 2002, Robinson 2005, WAIS 2006), alternative solutions have been explored. For instance, protein can be isolated from brain tissue previously used for RNA extraction using dialysis (Hummon, Lim et al. 2007), but is extensively time-consuming and difficult, and therefore limited to a small number of samples. Other methods previously described to enable simultaneous extraction of protein and RNA from a single tissue sample also have major limitations: Columns designed for this purpose (Tolosa, Schjenken et al. 2007) require sample incubation twice over night (at -20 °C, and then at 4 °C), during which protein composition may be susceptible to change (Zellner, Winkler et al. 2005); while tissue sonication in water (Leak, Castro et al. 2010) is not suitable for isolation of relatively labile products such as RNA, proteins containing post-translational modifications, or large protein complexes.

Here, we describe a simple and cheap method to homogenize frozen tissue into a fine powder such that a single brain structure, or specific combination of structures, can be used to obtain high-quality molecular products of any variety.

Methods

Subjects and tissue preparation

Care of animals and all protocols conformed to the guidelines set by the Veterinary Office of the Canton of Zurich, Switzerland, and approved by its Commission for Animal Research (150/2011). C57Bl/6J females (2½ months) were obtained from Elevage Janvier (Le Genest Saint Isle, France) and maintained in a temperature- and humidity-controlled facility on a 12 h reversed light/dark cycle with food and water *ad libitum*. Subjects were anesthetized with isoflurane and euthanized by decapitation. The brain was removed in ice-cold phosphate buffered saline (PBS; pH 7.4) and hippocampus isolated in the same solution. Left and right hippocampi were combined into a single pre-labeled microtube and immediately frozen in liquid N_2.

Materials

See Figure A-1 for a photo of the primary materials.

1. Large metal spatula
2. Solid metal block
3. Sterile, transparent bags
4. Small hammer
5. Scissors
6. Small metal spatula
7. Approximately two liters liquid N_2 in an insulated, wide-top container

Figure 1

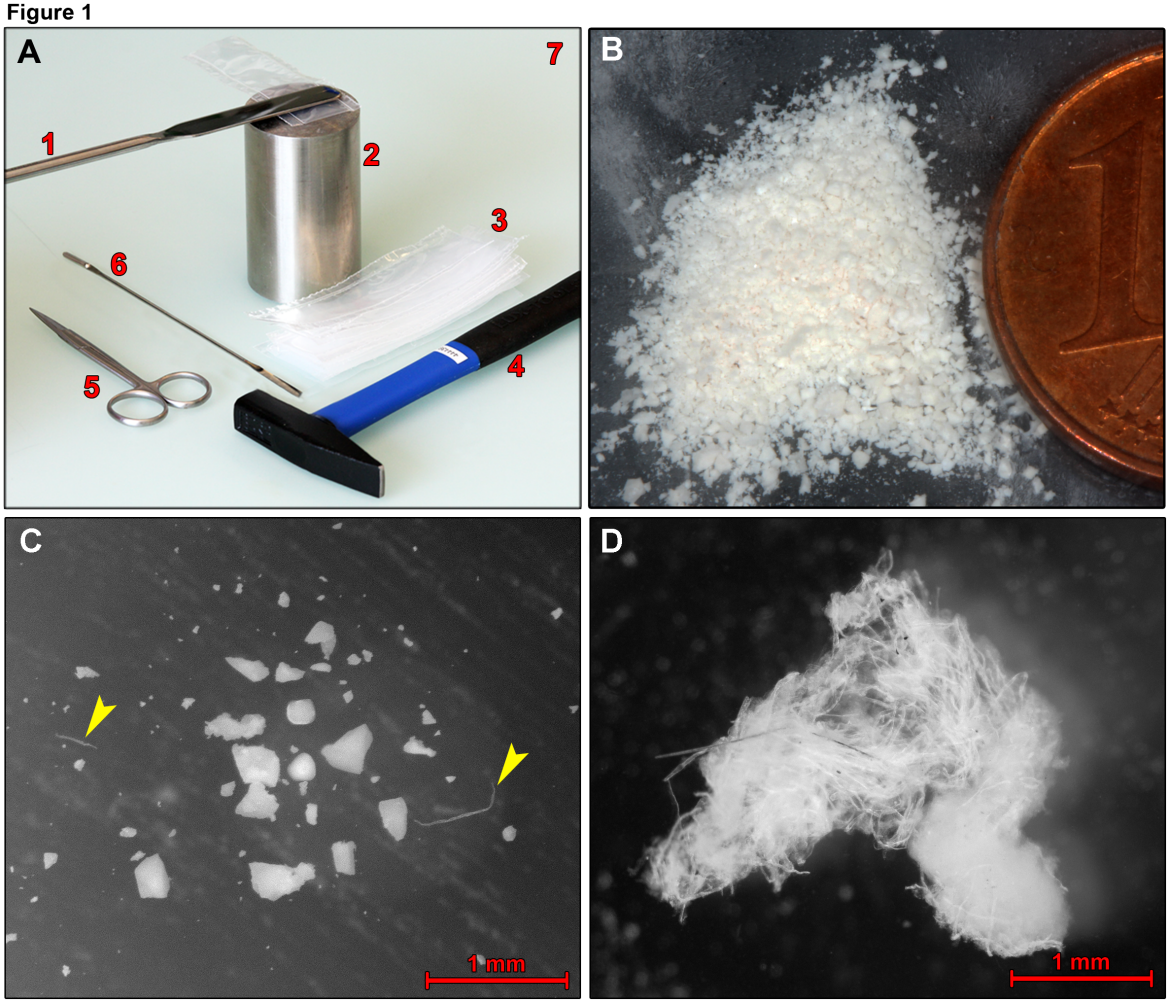

Figure A-1: Cryohomogenization equipment and cryohomogenized hippocampus powder. **(A)** Materials required for cryohomogenization. 1 – Large metal spatula. 2 – Solid metal block. 3 – Transparent bags, custom-sized in advance. 4 – Small hammer. 5 – Scissors. 6 – Small metal spatula. 7 – Liquid N_2 container and liquid N_2 (not shown). **(B and C)** Cryohomogenized hippocampus tissue powder with **(C)** presumed fragmented neural fibers (arrows). **(D)** Presumed neural fibers largely resistant to RIPA buffer homogenization with a needle alone (no cryohomogenization)

Cryohomogenization

Frozen brain tissue (whole mouse brain or isolated mouse hippocampi) was transferred to a sterile, pre-sized transparent bag and immediately placed on the top surface of a solid metal block pre-cooled in liquid N_2. Next, a large metal spatula, also pre-cooled in liquid N_2, was placed over the sample (as shown in Figure 1), and the tissue was crushed into a fine powder by gently hammering the large spatula overlying the brain tissue. Homogenized tissue powder was tapped into one corner of the bag, and the bag was cut open. Using a small pre-cooled metal spatula, the fine powder was allocated into three separate pre-cooled, pre-labeled and pre-weighed microtubes. Tubes were again weighed to determine tissue distribution. Using this approach, only the small metal spatula came in contact with the tissue sample, thereby minimizing the inter-sample cleaning time required to prevent cross-contamination. Individual sample processing time was approximately 1 min. Cryohomogenized hippocampus samples were used for abundance and quality assessment, in comparison to whole hippocampi.

RNA extraction and analysis

RNA was extracted with TRIzol in accordance with the manufacturer's instructions (Invitrogen; Carlsbad, CA). Briefly, 1 ml of TRIzol® RNA Isolation Reagent was added to each tube containing either whole frozen hippocampus, or cryohomogenized hippocampus powder, before being vortexed and past 20 times through either a 22G and then 26G needle (whole frozen hippocampi) or 26G needle alone (cryohomogenized hippocampus powder). After incubation at room temperature for 5 min, 200 µl of chloroform were added and the tubes were rigorously shaken for 15 s before incubation for a further 3 min and centrifugation (12000 g, 4 °C, 15 min). The aqueous phase overlying the organic and interphase was aspirated carefully and transferred to a fresh tube and processed for RNA isolation.

RNA quality and quantity were determined with nanodrop UV/V spectrophotometry, a 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA), and RT-qPCR, all according to the manufacturers' protocols. For RT-qPCR, Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA was compared to TATA-binding protein (*Tbp*) mRNA. Briefly, 1 µg of total RNA was

converted into cDNA using SuperScript®III Reverse Transcriptase (*Invitrogen*; Carlsbad, CA) and qPCR was performed using a LightCycler® 480 System (Roche; Basel, Switzerland) and LightCycler® 480 SYBR Green I Master (Roche; Basel, Switzerland). Primers for *Gapdh*: (forward: CACTGACGATCTCCCTCACA; reverse: GTGGGTGCAGCGAACTTTAT). Primers for *Tbpj*: (forward: GGGAGAATCATGGACCAGAA; reverse: TTGCTGCTGCTGTCTTTGTT). All RT-qPCRs were done in a 10 µl reaction volume containing 5 µl SYBR Green I Master, 2 µl ddH_2O, 1 µl primer pair (5 µM) and 2 µl of the cDNA (1:10 diluted).

Protein extraction and analysis

Protein extraction was performed according to standard laboratory practice with RIPA buffer (50 mM TRIS base; 150 mM NaCl; 1% Triton-X; 0.5% natrium deoxycholate; 0.1% SDS containing phenylmethylsulfonyfluorid (1:500), phosphatase inhibitor cocktails I, II & III (all 1:200) (Sigma-Aldrich; St. Louis, Missouri)). Briefly, 5 µl of RIPA was added for each mg of frozen whole hippocampus or cryohomogenized hippocampus powder, and past through sterile needles using the same procedure described above for TRizol extraction. The supernatant was collected in a fresh tube and used as the whole protein fraction. Protein yield was determined in triplicate by Bradford assay using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio Rad Laboratories AG; Reinach, BL, Switzerland).

Protein samples were also examined by western blot for GAPDH and β-Actin using SDS-Page and primary antibodies anti-GAPDH (14C10) Rabbit mAb (Cell Signaling; Beverly, MA) diluted 1:4000, and mouse anti-β-Actin diluted 1:10000 in TRIS buffered saline (TBS). Briefly, proteins were denaturated in SDS-loading buffer containing 10% mercaptoethanol by boiling for 5 min. 20 µg of total protein were loaded into each well of a 12% polyacrylamide gel. Following electrophoresis and transfer, the nitrocellulose membrane was blocked in 5% non-fat dry milk for 1 h at room temperature, washed 3x in TBS containing 0.5% tweet (TBS-T), incubated overnight at 4°C in primary antibody, washed 3x in TBS-T and incubated with goat anti-rabbit and goat anti-mouse secondary antibody diluted 1:10000 in TBS for 1 hour at room temperature. The membrane was visualized

with an ODYSSEY ® Imager (LI-COR Bioscience; Lincoln, Nebraska USA) and images analyzed using ImageJ (RSB web).

Statistical Analysis

Data were statistically examined by one-way or two-way analysis of variance (ANOVA) using Prism software (GraphPag Software, Inc., Irvine, California) version 5.0b.

Results

Cryohomogenization of frozen whole mouse brain or hippocampus

Frozen brain tissue processed according to the methods (*section 2.3*) were quickly transformed into a homogenous powder and kept frozen by close contact with the pre-cooled solid metal block. The powder consisted of particles approximately 10 to 100 µm in diameter. Individual sample processing time was about 1 min after becoming familiar with the procedure. Hippocampus tissue was used to investigate if cryohomogenization produced any change in product abundance or quality.

RNA abundance and quality is not significantly affected by cryohomogenization

We used standard laboratory methods to quantify and evaluate the quality of RNA either extracted from cryohomogenized hippocampi or whole frozen hippocampi. Nanodrop UV/V spectrophotometry did not demonstrate any significant difference in the amount of RNA isolated by either technique (Fig. A-2A). Moreover, both methods obtained comparable 260/280 ratios, suggesting a similar level of purity (Fig. A-2B). We also used a nucleic acid bioanalyzer to obtain flow cytometry plots of the RNA, but observed no major differences between the homogenization methods in any of the major peaks (Fig. A-2C). Finally, we produced cDNA libraries from the extracted RNA and performed RT-qPCR to quantify *Gapdh* and *Tbp* mRNA, and observed that both types of starting material yielded similar results, suggesting cryohomogenization does not impart limitations on cDNA synthesis or RT-qPCR (Fig. A-2D). Thus, common techniques use to analyze RNA are still effective following cryohomogenization.

Protein abundance and quality are not significantly affected by cryohomogenization

In the event that the cryohomogenization method somehow affected protein abundance, we used Bradford UV spectrometry but failed to observe any significant differences between the two types of starting material (Fig. A-2E). To investigate if protein quality was grossly affected, we also ran the protein extracts on a polyacrylamide gel, but found no visible changes by Coomassie blue staining (Fig. A-2F). Moreover, a nitrocellulose blot for the commonly used standard

proteins GAPDH and β-Actin demonstrated similar results following cryohomogenization compared to extraction from whole hippocampus (Fig. A-2G,H). Thus, common techniques use to analyze protein are as effective following cryohomogenization compared to protein extraction from whole tissue.

Figure 2

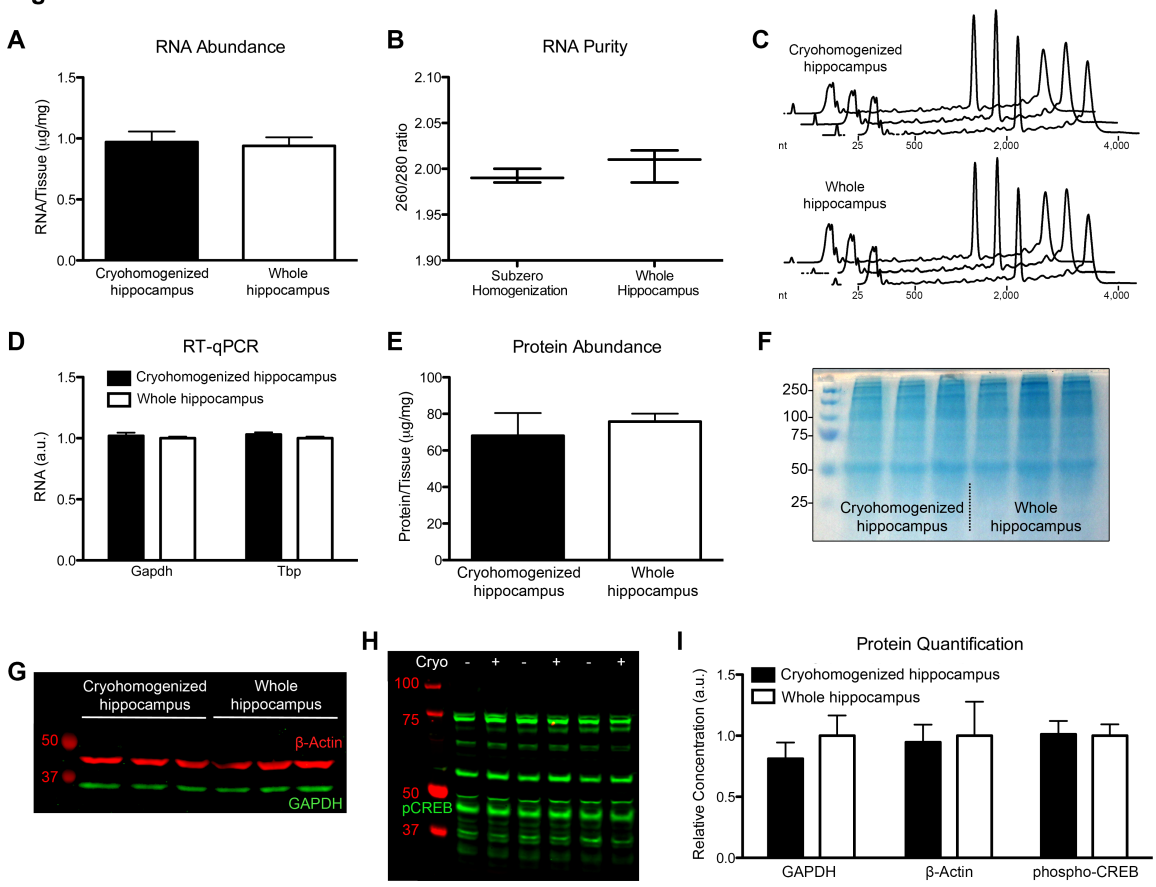

Figure A-2: RNA and protein abundance and quality is not significantly affected by cryohomogenization. **(A)** Nanodrop UV/V spectrophotometry demonstrates comparable total abundance and **(B)** 260/280 ratios for RNA obtained from cyrohomogenization hippocampus powder or whole frozen hippocampi. **(C)** Flow cytometry plots of the RNA extracted from either starting material shows no gross differences at peaks corresponding to 5S/tRNA, 18 S or 28 S rRNA. **(D)** RT-qPCR quantification of *Gapdh* and *Tbp* mRNA obtains similar results when cDNA libraries are created with RNA obtained from either cryohomogenized hippocampus powder or whole frozen hippocampi. **(E)** Bradford UV spectrometry did not demonstrate any significant difference between the two types of starting material, indicating no loss of protein occurred as a result of cryohomogenization. **(F)** Coomassie staining of the polyacrylamide membrane did not show gross changes as a result of cryohomogenization. **(G and H)** Western blot and **(I)** quantification of GAPDH, β-Actin and phospho-CREB were also similar following protein isolation from cryohomogenized powder or whole tissue. N = 3. Error bars, SEM.

Discussion

Neuroscience research at the molecular level frequently demands isolation of multiple distinct cellular products from a given brain region, leading experimentalists to designate one animal per desired molecular product. However, given the operational and ethic drawbacks to employing many animals, alternative methods that enable the parallel analysis of multifarious molecular classes would be more ideal. We describe here a simple, efficient and cheap method to cryohomogenize tissue into a fine powder that is then easily divided into proportions as desired for immediate molecular extraction or long-term storage. The current study demonstrates the feasibility for analyzing RNA and protein in parallel, but isolation of neurotransmitters, other small and large molecules, protein complexes, and proteins that require special extraction protocols (for example, synaptosomal or nucleic proteins, or proteins with elaborate post-translational modifications) could, in all probability, also be obtained with little, or no loss in abundance or quality.

The cryohomogenization method described in this report is conceptually similar to the use of a super-cooled mortar and pestle, but has the major advantage that only one small tool (the small metal spatula) must be cleaned between samples. This saves considerable time when performing multiple homogenizations. Moreover, by cryohomogenizing tissue within a custom-sized transparent bag, considerably less sample is lost compared to the mortar and pestle approach, thereby maximizing efficiency.

Several structures of the brain demonstrate bilateralization in experimental animals, including the hippocampus (Samara, Vougas et al. 2011), parietal cortex (Ogawa and Inui 2007), suprachiasmatic nucleus (Van der Zee, Roman et al. 2005), and amygdala (Carrasquillo and Gereau 2008, Kolber, Montana et al. 2010). For this reason, a given structure from one hemisphere may not contain a molecular organization comparable to the contralateral side. However, using the left hippocampus, for example, for protein extraction, and the right hippocampus, for example, for RNA isolation is clearly not ideal, though still sometimes performed. Cryohomogenization is therefore particularly appropriate for examining lateralized areas of the brain since the two structures (left and right) can be combined prior to homogenization, such that a more global readout is obtained

without compromising molecular abundance or the ability to analyze two (or more) distinct molecular classes.

Recent years have shown intensified effort to refine neuroscience techniques with the aim of reduce the number of experimental animals necessitated by a given experiment (Stokes, Kulpa-Eddy et al. 2012). While we previously demonstrated a refined approach to reduce the number of animals required to perform cognitive tasks (Saab, Saab et al. 2011), the focus of this work was to develop a means to reduce animal numbers required for molecular experiments.

In summary, we describe a method for tissue cryohomogenization with clear advantages over previously described techniques, and show that the resultant frozen powder is amenable to efficient multifarious molecular extraction.

Acknowledgements

B.J. Saab came up with the idea and directed the study. B. J. Saab and L. M. von Ziegler performed experiments and analyzed data. All authors wrote the manuscript. We thank Francesca Manuella and Johannes Bohacek for technical assistance. The lab of I.M. Mansuy is supported by the University of Zurich, the Swiss Federal Institute of Technology Zurich, the Swiss National Science Foundation, Roche, and the National Center of Competence in Research Neural Plasticity and Repair. B.J. Saab holds a 2012 NARSAD Young Investigator Award from the Brain & Behavior Research Foundation.

Appendix B

Early life epigenetic programming and transmission of stress-induced traits in mammals: how and when can environmental factors influence traits and their transgenerational inheritance?

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Summary

The environment can have a long-lasting influence on an individual's physiology and behavior. While some environmental conditions can be beneficial and result in adaptive responses, others can lead to pathological behaviors. Many studies have demonstrated that changes induced by the environment are expressed not only by the individuals directly exposed, but also by the offspring sometimes across multiple generations. Epigenetic alterations have been proposed as underlying mechanisms for such transmissible effects. Here, we review the most relevant literature on these changes and the developmental stages they affect the most. We discuss current evidence for transgenerational effects of prenatal and postnatal factors on bodily functions and behavioral responses, and the potential epigenetic mechanisms involved. We also discuss the need for a careful evaluation of the evolutionary importance with respect to health and disease, and possible directions for future research in the field.

Introduction

Changes in the environment induce behavioral adaptation

The ability to perceive and evaluate surrounding environments, and adopt appropriate behavioral responses is critical for living organisms (Zmigrod and Hommel 2013). It allows for suitable reaction to stimuli which increases the chance of survival and reproduction (Smith 2008). Maintaining a memory of such adaptive responses is essential for coping with similar conditions when encountered in later life (Mery 2013). Although behavioral adaptation is generally beneficial and helps adjust to a changing environment, it can also be maladaptive when external conditions and requirements change too rapidly and result in a mismatch with the adapted behavior/s (Daskalakis, Oitzl et al. 2012). Such divergence between an individual's response and the surrounding milieu can lead to inappropriate and pathological behaviors, and can increase the predisposition to disease (de Kloet, Joels et al. 2005). Thus, although an inherited trait is typically thought of as being beneficial and hence selected for, some inherited traits can be maladaptive in that they do not fit the progeny´s environmental demand (Daskalakis, Bagot et al. 2013). The biological mechanisms underlying adaptive behaviors are complex and involve activity-dependent changes in gene expression in multiple neural circuits and brain regions (de Kloet, Joels et al. 2005). Importantly, because these changes are modulated by the environment rather than being genetically encoded, many are mediated by non-genomic processes, in particular epigenetic mechanisms (Uchida, Hara et al. 2011, Klengel, Mehta et al. 2013)

The epigenetic code controls genomic activity

One of the primary functions of epigenetic processes is to remodel chromatin and thereby activate or silence genes. Chromatin comprises the DNA helix which wraps around octamers of histone proteins to form nucleosomes (Luger, Mader et al. 1997). It can be structurally remodeled by covalent modification of the DNA and histones, in particular DNA methylation (DNAme), and histone posttranslational modifications (HPTMs). The ensemble of these modifications constitutes an epigenetic code that alters gene activity without changing the genomic DNA sequence itself (Kouzarides 2007). In mammals, DNA methylation is a biochemical process that involves the covalent addition of a methyl group to cytosines in DNA,

preferentially onto CpG (cytosine-guanine) dinucleotides (Tost 2009). HPTMs are also covalent modifications that occur on protein histones in specific combinations and include, amongst others, acetylation, methylation (mono, bi or tri) phosphorylation and ubiquitylation (Agrawal, Laforsch et al. 1999, Tweedie-Cullen, Reck et al. 2009, Tweedie-Cullen, Brunner et al. 2012). The ensemble of modifications composed of DNAme and HPTMs establishes an epigenetic profile that is dynamically regulated at each individual gene. These marks modify the local electrochemical properties of chromatin, altering its conformation and thereby regulating the accessibility of genes to the transcriptional machinery (Allis 2007). Ultimately this modifies gene transcription in a spatial- and temporally-regulated manner in response to specific internal and external cues (Tsankova, Berton et al. 2006, Lister, Mukamel et al. 2013). Further to DNAme and HPTMs, increasing evidence has pointed to the importance of non-coding RNAs (ncRNAs) as an additional means of gene regulation. NcRNAs exist in a diverse range of sizes, and unlike messenger RNA (mRNA), are not translated into proteins but act to regulate gene expression. They can induce mRNA degradation and thereby downregulate protein translation, or they can act as guides of components of epigenetic machinery to specific DNA sequences (Agrawal, Laforsch et al. 1999, Ghildiyal and Zamore 2009, Di Ruscio, Ebralidze et al. 2013).

Epigenetic processes contribute to the transmission of acquired traits

Studies in rodents have shown that some epigenetic modifications in chromatin remodeling can persist and be maintained throughout life (Weaver, Cervoni et al. 2004, Roth, Lubin et al. 2009, Daskalakis, Oitzl et al. 2012). These modifications have the potential to be transmitted to subsequent generations if present in the germline (Skinner, Haque et al. 2013). The transmission of adaptive traits is an essential biological process that can have a tremendous impact on the evolution of a species (Agrawal, Laforsch et al. 1999). Although transmission provides an optimized response to an environment encountered by the previous generation, it has the potential to result in maladaptive behaviors if the environment changes in-between generations (Daskalakis, Bagot et al. 2013). Mechanistically, whilst the transgenerational inheritance of behaviors does not involve any change in the DNA sequence, it was nonetheless difficult to be explained conceptually via

epigenetic modifications. This is because most epigenetic marks, in particular DNAme, are erased from the chromatin during germ cell development and in the early zygote in mammals, a process known as epigenetic reprogramming. However at some genes, in particular imprinted genes and various other specific loci (Borgel, Guibert et al. 2010), epigenetic profiles can be maintained or re-instated despite reprogramming, and remain in the progeny. This strongly suggests that some, but perhaps not all, epigenetic profiles can persist across generations.

Here, we review the most recent evidence demonstrating that the acquisition of traits induced by environmental factors can occur during different developmental phases, that the acquired information can be transmitted across generations, and that it likely involves epigenetic mechanisms. We focus on traits induced by environmental changes in early life, their consequences on behavioral responses later in life and across subsequent generations.

The brain is susceptible to stress during critical periods in life

The influence of environmental factors on the body and underlying epigenetic mechanisms has been studied in relation to brain functions. In the brain, the (re)programming of epigenetic marks by environmental factors depends on cellular responses to intrinsic and extrinsic signals (Gapp, Woldemichael et al. 2012). It contributes to various brain processes and functions such as memory formation (Zovkic, Guzman-Karlsson et al. 2013), drug addiction (Nestler 2013) and stress responses (Hunter and McEwen 2013). In some cases, these marks are transient and dynamically regulated (Miller and Sweatt 2007, Day, Childs et al. 2013), whilst in others, they can persist and be perpetuated (Franklin, Russig et al. 2010). The strength and persistence of epigenetic changes strongly depend on the developmental stage and the time of establishment. The prenatal period (Kofman 2002), early childhood (Korosi and Baram 2010) and adolescence (Spear 2009) are critical temporal windows for the influence of environmental conditions in mammals. During these developmental phases, the brain experiences extensive growth (Finlay and Darlington 1995), remodeling (Lister, Mukamel et al. 2013), and is particularly sensitive to external conditions and interference (Andersen 2003).

Environments involving stress are especially detrimental. In humans, stressful conditions experienced during pregnancy increase the incidence of neurodevelopmental disorders such as schizophrenia and autism spectrum disorders in the child (van Os and Selten 1998, Khashan, Abel et al. 2008, Kinney, Miller et al. 2008). Likewise, in laboratory animals, such as rodents, gestational stress applied to the mother alters stress sensitivity, behavior, morphology, and gene expression in the resulting offspring (Mueller and Bale 2007, Mueller and Bale 2008). Environmental conditions in early postnatal life also strongly influence development, and increase predisposition to psychiatric disorders in later life in humans (Chu, Williams et al. 2013). This is as well the case in animals, in which the level of maternal care is particularly critical. Maternal nursing is directly associated with the formation of proper behavioral responses in later life, and the susceptibility to stress-induced disorders in adulthood. This link was shown to implicate epigenetic mechanisms of gene regulation, in particular, changes in DNAme of a regulatory region of the glucocorticoid receptor (*NR3C1*) in the hippocampus (Weaver, Cervoni et al. 2004). Adolescence is another critical window during which stress exposure can have detrimental consequences on mental health later in life. In humans, maltreatment during adolescence can induce antisocial behaviors in young adults (Smith, Ireland et al. 2005). In rodents, hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis due to stress during this period also alters behavioral responses and elicits multiple symptoms including increased aggression and antisocial behaviors (Marquez, Poirier et al. 2013, Veenit, Cordero et al. 2013).

The characteristics of stress exposure determine the consequences on brain and behavior

The impact and long-term consequences of stress exposure are known to depend on the type, severity and duration of the stressor(s). Stressors include a variety of environmental conditions such as psychological challenge and nutritional restriction.

Altered maternal care perturbs adult behaviors

The quality of the social and parental environment in early life is a critical determinant of the proper development of an individual. In humans, prolonged

separation from the mother and maternal neglect predispose an individual to behavioral deviance such as drug abuse in later life, in part by altering reward pathways (Enoch 2012). In rodent models, predictable maternal separation (subjected at the same time daily) often has no lasting behavioral effects in the offspring due to compensatory maternal behaviors (Macri, Mason et al. 2004). However, unpredictable and fragmented stress strongly compromises maternal sensory signals and triggers persistent cognitive and emotional dysfunctions in later life (Baram, Davis et al. 2012). In mice, unpredictable maternal separation combined with unpredictable maternal stress was shown to lead to a wide range of behavioral symptoms including depressive-like behaviors, social withdrawal, impaired social recognition and reduced risk assessment (Franklin, Russig et al. 2010, Franklin, Linder et al. 2011, Weiss, Franklin et al. 2011). Interestingly at the same time, this manipulation also increases behavioral flexibility and makes the animals more reactive in challenging situations (our own unpublished observations). This suggests that unpredictable stress in early life may provide some benefit later in life. In most cases, psychological stress acts as a negative factor, however under favorable conditions such as exposure to an enriched environment, beneficial effects may be observed (Nithianantharajah and Hannan 2006). Notably, the long-lasting effects of living conditions in early life have been reported to be sex-dependent. Whilst both females and males can be affected, the extent of behavioral alterations such as depressive-like behaviors can depend on gender (Dalla, Pitychoutis et al. 2011).

Malnutrition puts stress onto the organism

One of the first reports on the consequences of under-nutrition in humans is the effect of diet restriction during fetal life. A large-scale study in a Dutch cohort subjected to hunger during winter at the end of World War II (The Dutch Hunger Winter Families Study) showed that individuals born from mothers undernourished during pregnancy had altered epigenetic marks (Tobi, Lumey et al. 2009, Tobi, Slagboom et al. 2012). A differentially methylated region of the imprinted gene *IGF2* was shown to be hypomethylated in the blood of individuals born to these women up to 60 years after the period of hunger (Heijmans, Tobi et al. 2008). Many of these individuals suffered from metabolic alterations (Ravelli, van der

Meulen et al. 1998, Ravelli, van Der Meulen et al. 1999, Roseboom, van der Meulen et al. 2000) and a higher prevalence of psychiatric disorders including higher incidence of schizophrenia, and unipolar/bipolar depression (Susser, Neugebauer et al. 1996, Franzek, Sprangers et al. 2008, Stein, Pierik et al. 2009). In rodent models, malnutrition also alters behavior and impacts brain functions. Maternal high-fat diet during gestation increases anxiety and alters hippocampal serotonin level in mice (Peleg-Raibstein, Luca et al. 2012). It also reduces corticosterone and increases the level of its cognate receptors in the amygdala in the offspring (Sasaki, de Vega et al. 2013). Likewise in rats, direct exposure to a high-fat diet for an extended period (8 weeks) increases anxiety and corticosterone level (Buchenauer, Behrendt et al. 2009). However in contrast to long exposure, short exposure (1 week) to a high-fat diet has an opposite effect and is anxiolytic (Prasad and Prasad 1996).

The effects of environmental exposure can be passed to the following generation(s)

Numerous epidemiological and clinical studies in humans have underscored a strong heritable component in mood disorders like major depressive disorder (MDD) (Weissman, Wickramaratne et al. 2005), post traumatic stress disorder (Roberts, Galea et al. 2012) and associated externalizing and internalizing traits (Kim, Capaldi et al. 2009). However up to now, the heritability of these disorders could not be only attributed to genetic factors. Genes influencing such complex diseases have been proposed to contribute and act either as low penetrance common variants, or rare, highly penetrant inherited mutations. In the case of MDD, only approximately 40% of the risk was determined to be genetic (Sullivan, Neale et al. 2000), with the remaining 60% considered to be “missing heritability”. This “missing heritability” was postulated to be accounted for by environmental factors. Such factors may affect not only the exposed individuals but also their offspring, and thereby potentially impact several generations. This suggests that epigenetic changes brought about by the environment likely underlie some of the inheritance of complex diseases (Kendler 2001, Eichler, Flint et al. 2010, Millan, Agid et al. 2012). This hypothesis is strengthened by a recent epidemiological study showing that paternal obesity leads to *IGF2* hypomethylation in newborns

(Soubry, Schildkraut et al. 2013), suggesting that paternal malnutrition has an heritable influence on IGF2. Since IGF2 is a hormone that plays an essential role in promoting growth during gestation and is necessary for cognitive processes throughout life (Fowden, Sibley et al. 2006, Chen, Stern et al. 2011), it will be interesting to see whether the alterations in *IGF2* persist into adulthood and contribute to psychiatric disease risk.

Animal models have proven useful to study this question and the underlying mechanisms. Exposure to chronic traumatic stress during the first 2 weeks of life persistently alters behavioral responses across several generations in mice. Unpredictable maternal separation combined with unpredictable maternal stress in young mouse pups causes depressive-like behaviors and deficits in novelty response, risk assessment and social behaviors in adulthood (Franklin and Mansuy 2010, Franklin, Linder et al. 2011, Weiss, Franklin et al. 2011). These behavioral symptoms are transmitted to the following generation through both females and males (up to 3 generations for males) and are independent of maternal care. They are associated with alterations in DNAme in several stress-related genes in the adult brain, and sperm in first and second-generation animals, along with altered expression of these genes in the brain. Likewise in rats, adolescent stress has an impact across multiple generations. The offspring of stressed rat dams have increased anxiety but conversely also display better sociability and improved avoidance learning (Leshem and Schulkin 2012). Interestingly, exposure to an enriched environment before gestation has an effect on the offspring, opposite to that after stress exposure. The offspring of enriched dams show sex-dependent differences in anxiety level and reduced avoidance learning when compared to the offspring of stressed dams (Leshem and Schulkin 2012). Further, in juvenile mice (postnatal day 15 to 30), exposure to enriched conditions can rescue a deficit in synaptic plasticity in adulthood. Enrichment reverses a defect in hippocampal long-term potentiation (LTP), a form of synaptic plasticity linked to memory processes, in the exposed animals and also in the adolescent progeny of these animals (Arai, Li et al. 2009). Thus, traits acquired by environmental exposure have the potential to be transmitted across generations. Transmission may occur through different potential routes.

Potential routes of transmission of acquired traits across generations

Traits acquired by environmental exposure can be maintained and transferred from one generation to the next through different means. Some routes depend on the presence of the initial trigger i.e. poor maternal care, which is needed at each generation to reinstate the traits. Such routes are based on behavioral and social transfer. Other routes involve more stable mechanisms that become independent of the initial trigger, and reflect a molecular transfer implicating germ cells.

Behavioral and social transfer

Many traits acquired following exposure to environmental factors are transmitted from one generation to the next through behavioral and social interactions in early or adult life. In mammals, the quality and level of maternal care in early postnatal life have a strong influence on the progeny's development, and determine their physiological and behavioral responses in later life. In rats, maternal behaviors in mothers condition maternal behaviors in the female offspring. Thus, female rats providing insufficient maternal care give rise to female offspring that become poor mothers themselves. Mechanistically, such behavioral transfer is associated with broad epigenetic changes across the genome affecting multiple genes (McGowan, Suderman et al. 2011). Further, in rat, exposure of males to stressful anti-social experiences in youth increases aggression towards females in adulthood, an effect also observed in the offspring of these males. This transfer involves depressive behaviors of dams subjected to mistreatment by their mate, and also aggressive behaviors in the male offspring (Cordero, Poirier et al. 2012).

Molecular transfer

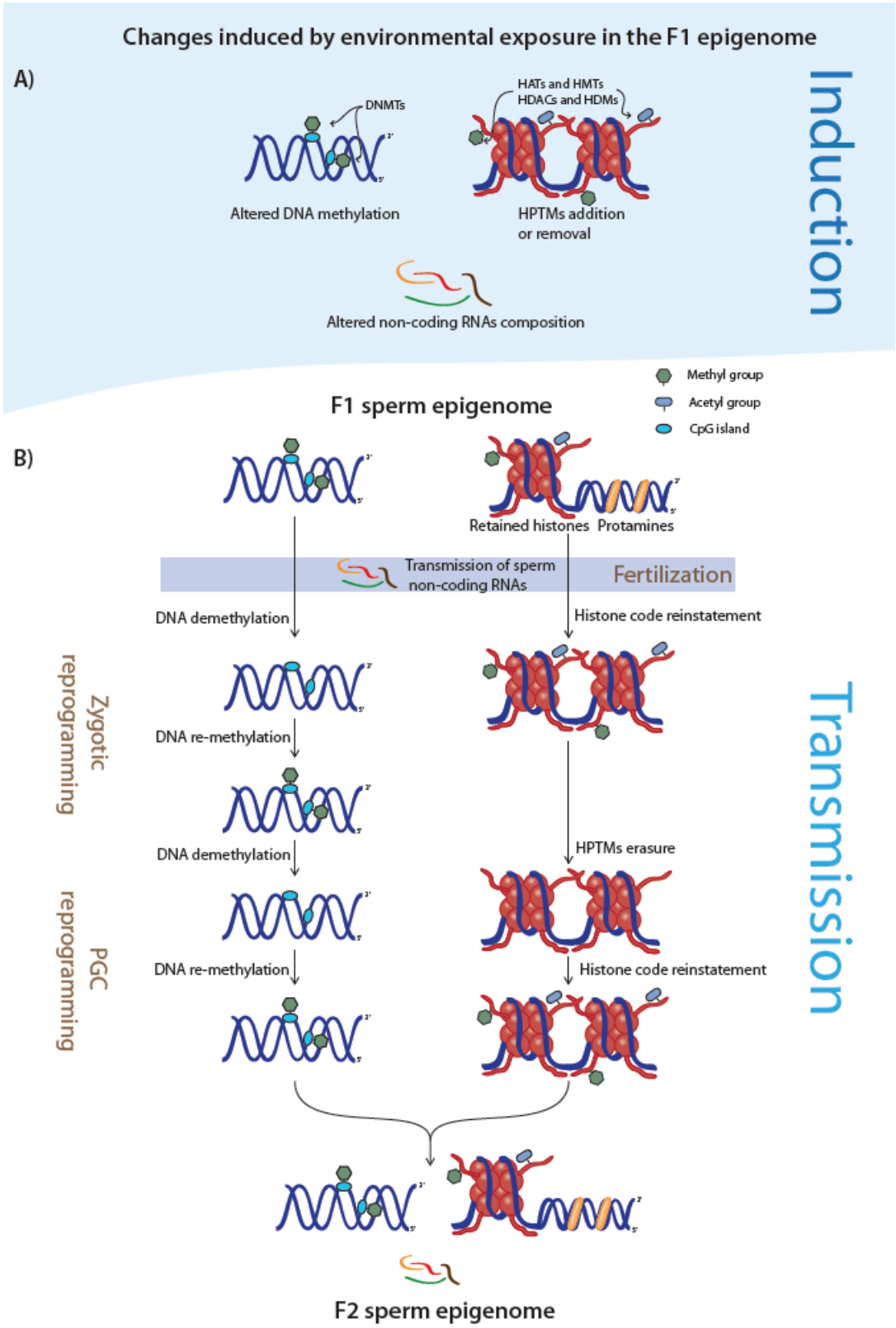
Pioneering studies in plants and invertebrates have provided initial insight into the potential mechanisms involved in epigenetic inheritance. Demonstrating that non-coding RNAs (ncRNAs) can act as carriers of information across generations and contribute to the transfer of acquired traits (Rechavi, Minevich et al. 2011, Ashe, Sapetschnig et al. 2012, Buckley, Burkhart et al. 2012, Grentzinger, Armenise et al. 2012). In mammals however, the mechanisms involved remain only partially elucidated. There are thought to be multiple mechanisms and that they depend on the developmental stage of induction. They determine the penetrance of the

effects and their perpetuation across subsequent generations. In this respect, a critical notion in transgenerational inheritance is the fact that inheritance can only be considered *truly transgenerational* and *epigenetic* if environmentally-induced traits do not need the initial trigger at each generation, and are observed in individuals of the third generation, whose founder germ cells have not been exposed to the trigger (Skinner 2008). The expression of the traits in these individuals is an indication that epigenetic mechanisms in germ cells are involved (Grossniklaus, Kelly et al. 2013). However, it is difficult to study these mechanisms in mammals, because germ cells are not easy to collect or to analyze. Further, ideally both maternal and paternal lines (matrilines and patrilines respectively) need to be examined. However, patrilines have the advantage of excluding maternal care confounds, possible social and/or behavioral transfer, and preventing interference by somatic components of oocytes and the *in utero* environment. Sperm cells are also more abundant than oocytes and easier to use for molecular analyses. However, since true epigenetic inheritance also occurs in matrilines (Weiss, Franklin et al. 2011), findings in male germ cells need to be validated in females. The following section discusses the importance of the developmental stage for the induction of persistent traits and presents various observations of the transmission of acquired traits. Although these findings are based on rodent studies, a mechanistic translation to humans can be envisaged given that the time window of epigenetic reprogramming in male germ cells relative to birth (pre- versus postnatal exposure), is comparable in mice and humans (De Felici 2013).

How epigenetic changes are transmitted across generations critically depends on the time of induction

The mere observation that an environmental condition induces epigenetic changes in the germline and specific traits in a subsequent generation does not guarantee true epigenetic inheritance. For true epigenetic inheritance, the epigenetic changes need to persist across generation. If the marks are not themselves maintained (for instance, a change in HPTMs in germ cells may only be transient), they need to be relayed by more stable and/or different marks. The induction and persistence of epigenetic changes is determined by the timing of the environmental exposure (Figure B-1). Although in theory, epigenetic changes can

occur throughout life, they are more likely to happen during early stages of development, in particular during epigenetic (re)programming of germ cells or in the embryo when the genome is in a malleable state (Liu, Balaraman et al. 2009).



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Zygotic epigenetic reprogramming

Epigenetic reprogramming engages a complex cascade of molecular events in early development that allows the dynamic establishment of epigenetic marks involving DNAme and HPTMs by successive waves of marking and erasure (Santos, Peters et al. 2005, Hajkova, Ancelin et al. 2008). In the early zygote, whilst the maternal and paternal genomes (derived from gametes) have different epigenetic profiles, they undergo zygotic reprogramming. DNAme marks are globally erased immediately post-fertilization and until the morula stage at preimplantation. In the female pronucleus, passive demethylation occurs upon consecutive cell divisions, correspondingly there is active demethylation in the male pronucleus (Santos, Hendrich et al. 2002). While DNA erasure affects genes globally, it spares a few of them, in particular imprinted genes, as well as genes expressed in the male germline (Borgel, Guibert et al. 2010), repeat-associated IAP retrotransposons (Lane, Dean et al. 2003) and genes in heterochromatin within and around centromeres (Oswald, Engemann et al. 2000). Further, soon after fertilization in the male pronucleus, protamines (histone-like proteins partially replacing histones during spermatogenesis) are exchanged with maternally-inherited histones (Adenot, Mercier et al. 1997). Subsequently, acetylation followed by methylation occurs on specific lysine (Lys, K) residues, for example K5 and K12 on H4 (Santos, Peters et al. 2005). Some maternal HPTMs established during oocyte growth, such as K9 and K27 methylation, are however maintained

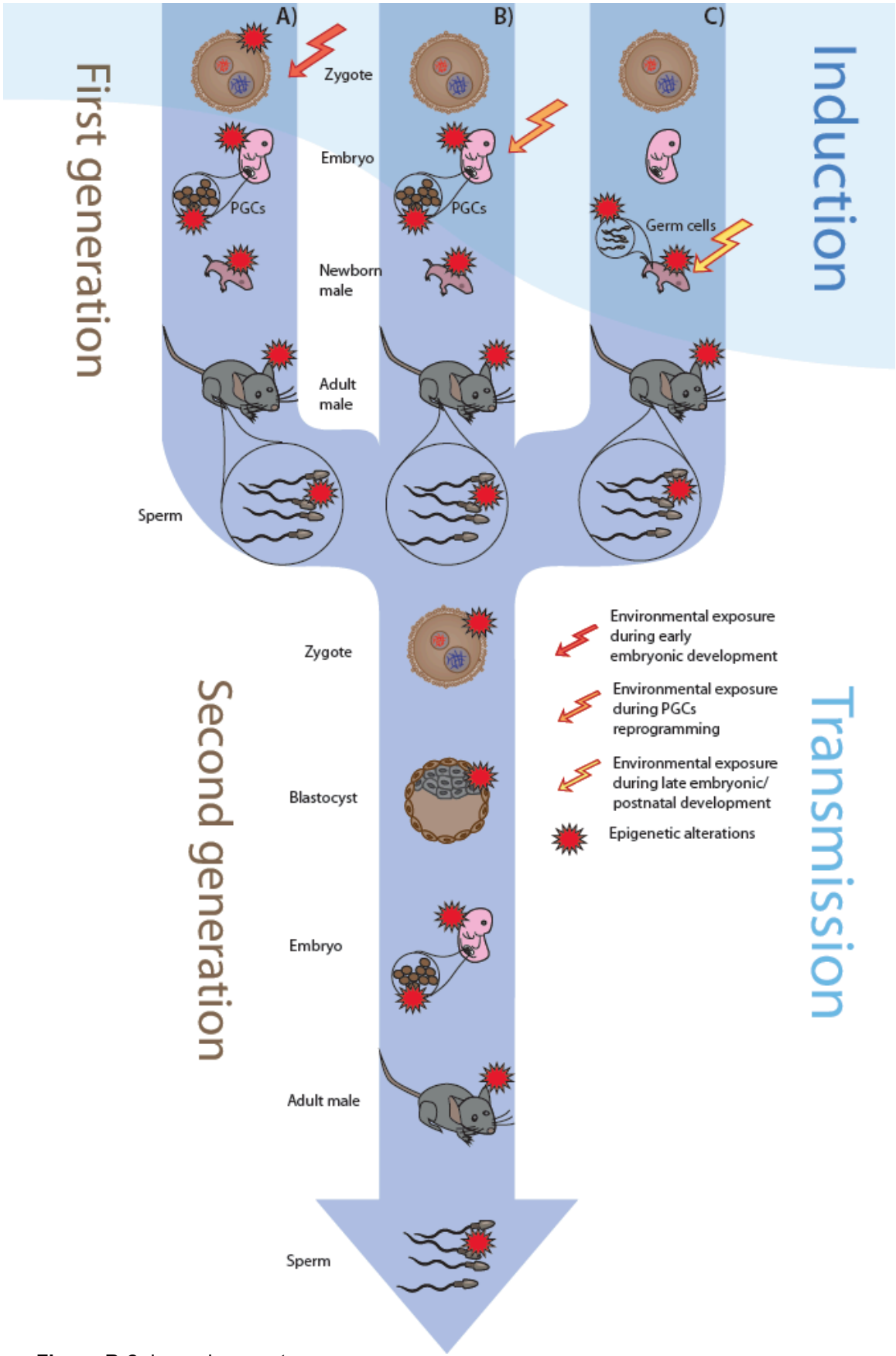
Figure B-1 (previous page): Induction and transmission of the effects of environmental exposure on the epigenome in rodents. **A**: Induction: Environmental factors can alter DNA methylation, HPTMs and the composition of ncRNA in animals exposed during either embryonic development, early post-natal life or adulthood,(F1). Multiple molecular modifiers can contribute to alterations in DNA methylation and HPTMs including DNMTs, which induce DNAme, and HATs/HMTs and HDACs/HDMs, which acetylate/methylate and deacetylate/demethylate histones respectively. DNAme and HPTMs alter the local properties of chromatin, such as the structure and charge, and thereby lead to changes in gene expression. **B**: Transmission: Some epigenetic marks may be maintained in germ cells during DNA and histone reprogramming from F1 to F2, and contribute to epigenetic inheritance. To be transmitted, DNAme marks must escape global erasure during fertilization, or be reinstated after erasure. Transmission of HPTMs also requires reinstatement of the histone code, which in germ cells, is complicated by the replacement of most histones by protamines during sperm maturation (Castillo, Simon et al. 2011). It therefore requires the selective retention of specific histones, or the reinstatement of the HPTMs in the zygote post-fertilization. Sperm ncRNAs that are delivered to the oocyte during fertilization may also contribute to this process (Rassoulzadegan, Grandjean et al. 2006, Rodgers, Morgan et al. 2013). For subsequent inheritance to F3, epigenetic alterations need to additionally resist the reprogramming that occurs in the F2 epigenome (in PGCs). Abbreviations: HPTMs, histone post translational modifications; DNAme, DNA methylation; DNMTs, DNA methyltransferases; ncRNA, non-coding RNA; HATs, histone acetyltransferases; HMTs, histone methyltransferases; HDACs, histone deacetylases; HDMs, histone demethylases.

(Santos, Peters et al. 2005) and therefore constitute an epigenetic memory.

Epigenetic reprogramming of primordial germ cells (PGCs)

Another wave of reprogramming takes place in primordial germ cells (PGCs), which are germ cell precursors in the early embryo. During this wave, DNAme and HPTMs (e.g. H3K9me2) are globally erased across the germ cell genome (Seki, Hayashi et al. 2005, Hajkova, Ancelin et al. 2008). But again, although most DNAme marks are erased, some are maintained at specific loci, for instance in genes containing or near repeat-associated IAP elements and in subtelomeric regions (Hackett, Sengupta et al. 2013). Imprinting is then established (Davis, Trasler et al. 1999, Bourc'his, Xu et al. 2001) to keep a parent-specific epigenetic mark and determine whether the maternal or paternal allele is expressed (Li, Beard et al. 1993). At a later stage of postnatal maturation in sperm, H4 variants also become hyperacetylated to allow nucleosome dissociation. Most histones are then substituted for protamines to allow for tighter packaging of the DNA (Castillo, Simon et al. 2011). However, some histones and their HPTMs, for instance H3K4me3 and H3K27me3, can be retained at loci containing developmental genes (Brykczynska, Hisano et al. 2010), and therefore provide another means to maintain epigenetic marks. Protamines in adult sperm can also carry multiple PTMs (Brunner 2014), suggesting the possibility that the histone-protamine transition or that protamine PTMs may contribute to information transfer from one generation to the next. Functionally, the successive waves of epigenetic reprogramming are paralleled by differential regulation of gene expression in the embryo (Aoki, Worrad et al. 1997). Transcription of both female and male genomes is increased at 2- and 4-cell stages but the male genome is more permissive to transcription during subsequent zygotic stages (Latham and Schultz 2001). Germ cell chromatin is therefore highly responsive during epigenetic reprogramming and is in a configuration susceptible to epigenetic alterations. The extent and persistence of alterations depend on the time of environmental exposure relative to epigenetic reprogramming. Whether a perturbation by environmental factors occurs shortly after fertilization, later in development or in adulthood, the impact and likelihood of transmission are different. Several time-

dependent scenarii for patriline inheritance can therefore be envisaged (Figure B-2) and thereby used to distinguish potential different mechanisms.



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Evidence for the involvement of different epigenetic mechanisms in the molecular transmission of acquired traits

Several studies have addressed the effect of environmental exposure during fetal development across multiple generations. Table 1 summarizes the evidence from rodent models for a transgenerational impact on brain and behavior, and indicates the mechanisms proposed to be implicated in the inheritance (Anway, Cupp et al. 2005, Skinner, Anway et al. 2008, Guerrero-Bosagna, Settles et al. 2010, Stouder and Paoloni-Giacobino 2010, Morgan and Bale 2011, Crews, Gillette et al. 2012, Wolstenholme, Edwards et al. 2012, Skinner, Haque et al. 2013). Many of these studies used exposure to an endocrine disruptor as trigger. It is conceivable that the induced mechanism(s) of inheritance does not differ from other detrimental exposures, and both may ultimately affect the stress system in the brain and thereby alter behavior. Hence, the transgenerational effects of endocrine disruptors can be viewed as an illustrative example for our purpose.

Inheritance of traits acquired by early postnatal, adolescent or adult environmental exposure have been observed in different conditions; some involving both patriline and matriline transmission (summarized in Table B-2 (Arai, Li et al. 2009, Roth, Lubin et al. 2009, Franklin, Russig et al. 2010, Dietz, LaPlant et al. 2011, Franklin, Linder et al. 2011, Weiss, Franklin et al. 2011, Guerrero-Bosagna, Covert et al. 2012, Leshem and Schulkin 2012, Rodgers, Morgan et al. 2013, Vassoler, White et al. 2013) with potential transmission mechanism). While DNAme, HPTMs and ncRNAs have all been proposed as potential transgenerational carriers of information, DNAme has been the most extensively explored (Anway, Cupp et al. 2005, Franklin, Russig et al. 2010, Guerrero-Bosagna, Settles et al. 2010, Stouder

Figure B-2 (previous page): Induction and transmission of environmental exposure during rodent development. **A**: Environmental exposure during early embryonic development, for instance before E10 in rodents, is likely to affect all somatic cells including future PGCs in the embryo. Such induction is most effective when it occurs in the zygote through to the blastocyst (between E0 and E3.5) in the first generation. This is because the chromatin is reprogrammed during this stage and is therefore more susceptible to alterations (Santos, Hendrich et al. 2002). **B:** Environmental exposure between E10 and E13 may perturb proper PGC reprogramming, and epigenetic marks that resist zygotic reprogramming after fertilization, which is not as extensive as PGC reprogramming (Oswald, Engemann et al. 2000, Lane, Dean et al. 2003), are present in the individuals derived from these germ cells. **C:** Environmental exposure during late embryogenesis and postnatal development can also induce heritable epigenetic changes in germ cells, although germ cells at this stage of development are less susceptible to interference. In A, B and C, true transgenerational transmission requires that epigenetic changes persist through both germ cell and zygotic reprogramming. Abbreviations: E, embryonic day; PGC, primordial germ cell.

and Paoloni-Giacobino 2010, Skinner, Haque et al. 2013) (see Tables B-1 and B-2). Environmental exposure impacting imprinted genes is particularly interesting since the mechanisms operating to protect these genes from reprogramming (Hirasawa, Chiba et al. 2008, Ciccone, Su et al. 2009) may be recruited for non-genomic inheritance of acquired traits. Studies on vinclozolin or stress exposure have indeed shown that imprinted genes can be affected (Franklin, Russig et al. 2010, Stouder and Paoloni-Giacobino 2010), suggesting a susceptibility of these genes to environmental changes. However, susceptibility decreases for exposure after establishment of imprinting in PGCs (Radford, Isganaitis et al. 2012). Future studies should determine whether these genes might predispose higher susceptibility of PGCs to environmental changes during imprinting. Further, although no substantial reprogramming takes place in the male germline during postnatal life, epigenetic marks continue to be established during this period (Sasaki and Matsui 2008), making them a target for interference. In agreement, studies in our lab have shown that imprinted genes can be affected postnatally (Franklin, Russig et al. 2010). It has been suggested that environmental exposure could put epigenetic modifications of non-imprinted genes in an 'imprinted-like' state and thereby enable their transmission (Oswald, Engemann et al. 2000, Lane, Dean et al. 2003, Borgel, Guibert et al. 2010, Hackett, Sengupta et al. 2013).

Mechanistically, the inheritance of traits acquired after birth (Table B-1) may also involve pathways different from those during embryogenesis (Table B-2). While studies of embryonic exposure only provide evidence for the involvement of DNAme in transmission, later exposure may implicate other epigenetic modifications such as HPTMs and ncRNAs (See Tables B-1 and B-2). Thus, histones and protamines both carry PTMs, and histones have recently been implicated in the inheritance of the effects of cocaine self-administration in male rats (Vassoler, White et al. 2013). Further, ncRNAs are abundant in sperm cells and may be altered by external factors. Indeed, initial evidence points to the possible involvement of small ncRNAs in the transmission of stress-induced traits. For instance, exposure to chronic stress for 6 weeks during puberty or adulthood alters a pool of miRNA in sperm, and reduces HPA axis responsiveness in the offspring. Unpredictable traumatic stress in early postnatal life also alters miRNA

content in mouse sperm (Rodgers, Morgan et al. 2013), and has effects across generations that are associated with sperm RNAs (our own unpublished observations). However, how sperm RNAs are involved in the transmission of stress effects still needs to be determined. These results corroborate previous data showing that sperm RNAs can underlie the transmission of a genetically-induced phenotype (Rassoulzadegan, Grandjean et al. 2006).

Table B-1:

Effects of prenatal enviromental exposure on subsequent generations									
Study	Model organism	Environmental exposure	Timing	Behavioral alterations	Physiological alterations	Epigenetic mechanism involved	Breeding modality	Generations investigated	True epigenetic inheritance
Anway et al. 2005	rat	Vinclozolin	E8-E15		DNAme in testis of F1 males; decreased spermatogenic capacity	DNAme	interbreeding of descendents	1,2,3,4	yes
Skinner et al. 2008	rat	Vinclozolin	E8-14	sex-specific anxiety behavior in F3	sex-specific alteration in hippocampal gene expression		outbred; inter breeding of descendents	3	yes
Guerrero-Bosagna et al. 2010	rat	Vinclozolin	E8-E14		DNAme at the promoter of most genes that contain a specific consensus sequence in the germline	DNAme	outbred; inter breeding of descendents	3	yes
Stouder et al. 2010	rat	Vinclozolin	E10-E18		altered DNAme at imprinted genes in tail, sperm, liver, skeletal muscle; decreased motile sperm in F1	DNAme	male line	1,2,3	yes
Morgan et al. 2011	mice	chronic stress	E1-E7	increased stress sensitivity in F1, increased depressive-like behavior and decreased anogenital distance in F2 males	hormonal regulation, dysmasculinatio n of neurodevelopme ntal gene expression and miRNA expression in F2		male line	1,2	no
Crews et al. 2012	rat	Vinclozolin	E8-14	altered anxiety in response to stress in F3	overall altered metabolic activity in brain, altered testosterone level in response to stress, altered hippocampal gene expression		non-littermate inter breeding of descendents	3	yes
Wolstenholme et al. 2012	rat	Bisphenol A	gestation	altered sociability down to F4	estrogen receptor, oxytocin and vasopressin expression		outbred; for F1: crossfostering to control mothers, for F2: brother-sister pairing	1,2,3,4	yes
Guerrero-Bosagna et al. 2012	mouse	Vinclozolin	E7-E13		spermatogenic cell defects, testicule, prostate and kidney abnormalities, polycystic ovarian disease only in outbred descendents		outbred and inbred strain, no littemate inbreeding	3	yes
Skinner et al. 2013	rat	Vinclozolin	E8-E14		germline	DNAme	outbred strain, inter breeding of descendents	3	yes

Table B-2:

Effects of postnatal environmental exposure on subsequent generations									
Study	Model organism	Environmental exposure	Timing	Behavioral alterations	Physiological alterations	Epigenetic mechanism involved	Breeding modality	Generations investigated	True epigenetic inheritance
Arai et al. 2009	mouse	environmental enrichment w/wo mutant background	postnatal week 2-4	increased brain plasticity, increased learning			female line with crossfostering for mutant background	1,2	no
Roth et al. 2009	rat	aversive maternal care	PND1-PND7		altered BDNF gene methylation and expression in prefrontal cortex		female line with crossfostering	1,2	no
Franklin et al. 2010	mouse	unpredictable maternal separation combined with unpredictable maternal stress	PND1-PND14	depressive-like behavior, altered approach avoidance behavior	altered gene expression and DNAme at the promoter of stress related genes and MeCP2 in F2 hippcocampus, and F1 and F2 sperm	DNAme	male line	1,2,3,	yes
Franklin et al. 2011	mouse	unpredictable maternal separation combined with unpredictable maternal stress	PND1-PND14	alterations in sociability in F2 and F3, in social recognition in F1, F2 and F3, altered response to social defeat in F2	altered 5HT1AR binding and serotonin level in the brain		male line	1,2,3	yes
Weiss et al. 2011	mouse	unpredictable maternal separation alone or combined with unpredictable maternal stress	PND1-PND14	depressive-like behavior in F1, altered approach avoidance behavior in F1 and F2	altered CRFR2 binding in the brain in F1		female line with crossfostering	1,2	no
Dietz et al. 2011	mouse	chronic social defeat	10 days in adulthood	depressive-like and anxiety behavior	sex-specific increase in corticosterone, decrease in vascular endothelial growth factor in F2		male line & IVF using naive oocytes	1,2	no
Leshem et al. 2012	rat	environmental enrichment and or mild stress	stress: PND27-29; EE: PND21 - PND60	avoidance learning, anxiety, sex-specific effect on accoustic startle test, decreased social interaction in males			female line	2	no
Rodgers et al. 2013	mouse	chronic stress	6 weeks during adolescence		reduced HPA axis responsiveness	altered miRNAs in sperm	male line	2	no
Vassoler et al. 2013	rat	cocaine self-administration	60 days in adulthood	sex-specific cocaine resistance	increased BDNF gene expression and acetylation in the medial prefrontal cortex, altered H3 acetylation in the germline of fathers	retained histone PTMs	male line	1,2	no

Conclusions

Environmental exposure can have long lasting effects on brain and behavior that can persist over several generations. The mechanisms underlying such transgenerational transmission involve epigenetic processes, which enable the stable transfer of the molecular basis of acquired traits. Despite some reports of molecular transfer or true transgenerational inheritance of acquired traits, these mechanisms remain mostly unknown. This is in part due to their complexity and the difficulty of studying them in animal models, and certainly in humans. Therefore, the analysis of these mechanisms first requires the establishment of robust, consistent and reliably transmitted phenotypic traits in a model system. Then, timely and targeted measurement of epigenetic marks in the right tissue or cells, and on the specific genes or loci is also required, with proper timing of environmental exposure. So far, most studies have used models with a broad timing of exposure (several days to several weeks) and a single time-point as the read-out of epigenetic alterations. These studies have therefore not allowed to determine the most critical time window of induction, nor the time course of epigenetic changes. Moreover, in addition to DNAme, HPTMs and sncRNAs, other non-genomic processes such as 5-hydroxy-DNAme, RNA methylation, long non-coding RNAs would also be interesting to examine. Clearly, such processes and mechanisms are likely intertwined with genetic factors, and studies considering genome-epigenome interactions will be necessary. The use of novel techniques and methodologies such as high-throughput epigenetic screening and molecular imaging are expected to facilitate a better understanding of these mechanisms, and of their functional and evolutionary impact (Wan, Gu et al. 2013).

References

Adenot, P. G., et al. (1997). "Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos." Development **124**(22): 4615-4625.

Aebersold, R. and M. Mann (2003). "Mass spectrometry-based proteomics." Nature **422**(6928): 198-207.

Agrawal, A. A., et al. (1999). "Transgenerational induction of defences in animals and plants." Nature **401**(6748): 60-63.

Allis, C. D. J., T; Reinberg, D. (2007). Epigenetics, Cold Spring Harbor Laboratory Press.

Anderse, P., et al. (2006). "The Hippocampus Book." Oxford Neuroscience.

Andersen, P., et al. (1966). "Location and identification of excitatory synapses on hippocampal pyramidal cells." Exp Brain Res **1**(3): 236-248.

Andersen, S. L. (2003). "Trajectories of brain development: point of vulnerability or window of opportunity?" Neurosci Biobehav Rev **27**(1-2): 3-18.

Anway, M. D., et al. (2005). "Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility." Science **308**(5727): 1466-1469.

Aoki, F., et al. (1997). "Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo." Dev Biol **181**(2): 296-307.

Arai, J. A., et al. (2009). "Transgenerational rescue of a genetic defect in long-term potentiation and memory formation by juvenile enrichment." J Neurosci **29**(5): 1496-1502.

Ashe, A., et al. (2012). "piRNAs can trigger a multigenerational epigenetic memory in the germline of C. elegans." Cell **150**(1): 88-99.

Au, H. K., et al. (2005). "Calcium-dependent up-regulation of mitochondrial electron transfer chain gene expressions in human luteinized granulosa cells." Fertil Steril **84 Suppl 2**: 1104-1108.

Balbuena, T. S., et al. (2014). "Global quantitative proteomics using spectral counting: an inexpensive experimental and bioinformatics workflow for deep proteome coverage." Methods Mol Biol **1072**: 171-183.

Bannerman, D. M., et al. (2003). "Ventral hippocampal lesions affect anxiety but not spatial learning." Behav Brain Res **139**(1-2): 197-213.

Baram, T. Z., et al. (2012). "Fragmentation and unpredictability of early-life experience in mental disorders." Am J Psychiatry **169**(9): 907-915.

Beran, M. J., et al. (2016). "Primate cognition: attention, episodic memory, prospective memory, self-control, and metacognition as examples of cognitive control in nonhuman primates." Wiley Interdiscip Rev Cogn Sci **7**(5): 294-316.

Berlyne, D. E. (1950). "Novelty and curiosity as determinants of exploratory behaviour." British Journal of Psychology **41**(1-2): 68-80.

Binder, J. R. and R. H. Desai (2011). "The neurobiology of semantic memory." Trends Cogn Sci **15**(11): 527-536.

Birsoy, K., et al. (2015). "An Essential Role of the Mitochondrial Electron Transport Chain in Cell Proliferation Is to Enable Aspartate Synthesis." Cell **162**(3): 540-551.

Borgel, J., et al. (2010). "Targets and dynamics of promoter DNA methylation during early mouse development." Nat Genet **42**(12): 1093-1100.

Borger, E., et al. (2014). "The calcium-binding protein EFhd2 modulates synapse formation in vitro and is linked to human dementia." J Neuropathol Exp Neurol **73**(12): 1166-1182.

Borovok, N., et al. (2016). "Dynamics of Hippocampal Protein Expression During Long-term Spatial Memory Formation." Mol Cell Proteomics **15**(2): 523-541.

Bourc'his, D., et al. (2001). "Dnmt3L and the establishment of maternal genomic imprints." Science **294**(5551): 2536-2539.

Broadbent, N. J., et al. (2010). "Object recognition memory and the rodent hippocampus." Learn Mem **17**(1): 5-11.

Brown, M. W., et al. (2010). "Recognition memory: material, processes, and substrates." Hippocampus **20**(11): 1228-1244.

Brunner, A. M., Nanni, P. Mansuy I.M. (2014). "Epigenetic marking of sperm by post-translational modification of histones and protamines." Epigenetics and Chromatin.

Brykczynska, U., et al. (2010). "Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa." Nat Struct Mol Biol **17**(6): 679-687.

Buchenauer, T., et al. (2009). "Diet-induced obesity alters behavior as well as serum levels of corticosterone in F344 rats." Physiol Behav **98**(5): 563-569.

Buckley, B. A., et al. (2012). "A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality." Nature **489**(7416): 447-451.

Bunsey, M. and H. Eichenbaum (1996). "Conservation of hippocampal memory function in rats and humans." Nature **379**(6562): 255-257.

Buwalda, B., et al. (2005). "Long-term effects of social stress on brain and behavior: a focus on hippocampal functioning." Neurosci Biobehav Rev **29**(1): 83-97.

Cai, D. J., et al. (2016). "A shared neural ensemble links distinct contextual memories encoded close in time." Nature **534**(7605): 115-118.

Cajigas, I. J., et al. (2010). "Protein homeostasis and synaptic plasticity." EMBO J **29**(16): 2746-2752.

Carrasquillo, Y. and R. W. t. Gereau (2008). "Hemispheric lateralization of a molecular signal for pain modulation in the amygdala." Molecular pain **4**: 24.

Castillo, J., et al. (2011). "Protamine/DNA ratios and DNA damage in native and density gradient centrifuged sperm from infertile patients." J Androl **32**(3): 324-332.

Cembrowski, M. S., et al. (2016). "Hipposeq: a comprehensive RNA-seq database of gene expression in hippocampal principal neurons." Elife **5**: e14997.

Chen, D. Y., et al. (2011). "A critical role for IGF-II in memory consolidation and enhancement." Nature **469**(7331): 491-497.

Chiba, A. A., et al. (1994). "Memory for spatial location as a function of temporal lag in rats: role of hippocampus and medial prefrontal cortex." Behav Neural Biol **61**(2): 123-131.

Cho, Y. H. and R. P. Kesner (1995). "Relational object association learning in rats with hippocampal lesions." Behav Brain Res **67**(1): 91-98.

Choi, M., et al. (2014). "MSstats: an R package for statistical analysis of quantitative mass spectrometry-based proteomic experiments." Bioinformatics **30**(17): 2524-2526.

Chu, D. A., et al. (2013). "Early life trauma predicts self-reported levels of depressive and anxiety symptoms in nonclinical community adults: Relative contributions of early life stressor types and adult trauma exposure." J Psychiatr Res **47**(1): 23-32.

Ciccone, D. N., et al. (2009). "KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints." Nature **461**(7262): 415-418.

Cohen, S. J. and R. W. Stackman, Jr. (2015). "Assessing rodent hippocampal involvement in the novel object recognition task. A review." Behav Brain Res **285**: 105-117.

Conway, M. A. (2009). "Episodic memories." Neuropsychologia **47**(11): 2305-2313.

Cordero, M. I., et al. (2012). "Evidence for biological roots in the transgenerational transmission of intimate partner violence." Transl Psychiatry **2**: e106.

Costa-Mattioli, M. and N. Sonenberg (2008). "Translational control of gene expression: a molecular switch for memory storage." Prog Brain Res **169**: 81-95.

Costenoble, R., et al. (2011). "Comprehensive quantitative analysis of central carbon and amino-acid metabolism in Saccharomyces cerevisiae under multiple conditions by targeted proteomics." Mol Syst Biol **7**: 464.

Crews, D., et al. (2012). "Epigenetic transgenerational inheritance of altered stress responses." Proceedings of the National Academy of Sciences **109**(23): 9143-9148.

Crystal, J. D. (2010). "Episodic-like memory in animals." Behav Brain Res **215**(2): 235-243.

Csicsvari, J., et al. (2007). "Place-selective firing contributes to the reverse-order reactivation of CA1 pyramidal cells during sharp waves in open-field exploration." Eur J Neurosci **26**(3): 704-716.

Dalla, C., et al. (2011). "Sex differences in response to stress and expression of depressive-like behaviours in the rat." Curr Top Behav Neurosci **8**: 97-118.

Daskalakis, N. P., et al. (2013). "The three-hit concept of vulnerability and resilience: Toward understanding adaptation to early-life adversity outcome." Psychoneuroendocrinology **38**(9): 1858-1873.

Daskalakis, N. P., et al. (2012). "Testing the cumulative stress and mismatch hypotheses of psychopathology in a rat model of early-life adversity." Physiol Behav **106**(5): 707-721.

Davis, T. L., et al. (1999). "Acquisition of the H19 methylation imprint occurs differentially on the parental alleles during spermatogenesis." Genomics **58**(1): 18-28.

Day, J. J., et al. (2013). "DNA methylation regulates associative reward learning." Nat Neurosci.

Day, M., et al. (2003). "Glutamate-receptor-mediated encoding and retrieval of paired-associate learning." Nature **424**(6945): 205-209.

De Felici, M. (2013). Oogenesis, Origin, Migration and Proliferation of Human primordial Germ Cells, Springer-Verlag-London.

de Kloet, E. R., et al. (2005). "Stress and the brain: from adaptation to disease." Nat Rev Neurosci **6**(6): 463-475.

Dere, E., et al. (2006). "The case for episodic memory in animals." Neurosci Biobehav Rev **30**(8): 1206-1224.

Di Ruscio, A., et al. (2013). "DNMT1-interacting RNAs block gene-specific DNA methylation." Nature **503**(7476): 371-376.

Dickerson, B. C. and H. Eichenbaum (2010). "The episodic memory system: neurocircuitry and disorders." Neuropsychopharmacology **35**(1): 86-104.

Dietz, D. M., et al. (2011). "Paternal Transmission of Stress-Induced Pathologies." Biol Psychiatry **70**(5): 408-414.

Dowle, A. A., et al. (2016). "Comparing the Diagnostic Classification Accuracy of iTRAQ, Peak-Area, Spectral-Counting, and emPAI Methods for Relative Quantification in Expression Proteomics." J Proteome Res **15**(10): 3550-3562.

Dudek, S. M., et al. (2016). "Rediscovering area CA2: unique properties and functions." Nat Rev Neurosci **17**(2): 89-102.

Eichenbaum, H. (2004). "Hippocampus: cognitive processes and neural representations that underlie declarative memory." Neuron **44**(1): 109-120.

Eichenbaum, H., et al. (1999). "The hippocampus, memory, and place cells: is it spatial memory or a memory space?" Neuron **23**(2): 209-226.

Eichenbaum, H., et al. (1987). "Cue-sampling and goal-approach correlates of hippocampal unit activity in rats performing an odor-discrimination task." J Neurosci **7**(3): 716-732.

Eichler, E. E., et al. (2010). "Missing heritability and strategies for finding the underlying causes of complex disease." Nat Rev Genet **11**(6): 446-450.

Ekstrand, M. and N. G. Larsson (2002). "Breeding and genotyping of Tfam conditional knockout mice." Methods Mol Biol **197**: 391-400.

Eldridge, L. L., et al. (2000). "Remembering episodes: a selective role for the hippocampus during retrieval." Nat Neurosci **3**(11): 1149-1152.

Enoch, M. A. (2012). "The influence of gene-environment interactions on the development of alcoholism and drug dependence." Curr Psychiatry Rep **14**(2): 150-158.

Ernst, J. and Z. Bar-Joseph (2006). "STEM: a tool for the analysis of short time series gene expression data." BMC Bioinformatics **7**: 191.

Ernst, J. and M. Kellis (2010). "Discovery and characterization of chromatin states for systematic annotation of the human genome." Nat Biotechnol **28**(8): 817-825.

Ernst, J., et al. (2005). "Clustering short time series gene expression data." Bioinformatics **21 Suppl 1**: i159-168.

Escandon, A., et al. (2010). "Effect of cognitive fluctuation on neuropsychological performance in aging and dementia." Neurology **74**(3): 210-217.

Finlay, B. L. and R. B. Darlington (1995). "Linked regularities in the development and evolution of mammalian brains." Science **268**(5217): 1578-1584.

Flecknell, P. (2002). "Replacement, reduction and refinement." ALTEX **19**(2): 73-78.

Fortin, N. J., et al. (2002). "Critical role of the hippocampus in memory for sequences of events." Nat Neurosci **5**(5): 458-462.

Fowden, A. L., et al. (2006). "Imprinted genes, placental development and fetal growth." Horm Res **65 Suppl 3**: 50-58.

Franklin, T. B., et al. (2011). "Influence of Early Stress on Social Abilities and Serotonergic Functions across Generations in Mice." PLoS One **6**(7): e21842.

Franklin, T. B. and I. M. Mansuy (2010). "Epigenetic inheritance in mammals: Evidence for the impact of adverse environmental effects." Neurobiology of Disease **39**(1): 61-65.

Franklin, T. B., et al. (2010). "Epigenetic transmission of the impact of early stress across generations." Biol Psychiatry **68**(5): 408-415.

Franz, A., et al. (2015). "Double-edged alliance: mitochondrial surveillance by the UPS and autophagy." Curr Opin Cell Biol **37**: 18-27.

Franzek, E. J., et al. (2008). "Prenatal exposure to the 1944-45 Dutch 'hunger winter' and addiction later in life." Addiction **103**(3): 433-438.

Frey, D., et al. (2000). "Shared and unique roles of CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomical plasticity." J Cell Biol **149**(7): 1443-1454.

Gal-Ben-Ari, S., et al. (2012). "Consolidation and translation regulation." Learn Mem **19**(9): 410-422.

Gapp, K., et al. (2012). "Epigenetic regulation in neurodevelopment and neurodegenerative diseases." Neuroscience.

Gasbarri, A., et al. (1997). "The dopaminergic mesencephalic projections to the hippocampal formation in the rat." Prog Neuropsychopharmacol Biol Psychiatry **21**(1): 1-22.

Ghandhi, S. A., et al. (2011). "Time-series clustering of gene expression in irradiated and bystander fibroblasts: an application of FBPA clustering." BMC Genomics **12**: 2.

Ghildiyal, M. and P. D. Zamore (2009). "Small silencing RNAs: an expanding universe." Nat Rev Genet **10**(2): 94-108.

Gil, O. D., et al. (1998). "Neurotrimin mediates bifunctional effects on neurite outgrowth via homophilic and heterophilic interactions." J Neurosci **18**(22): 9312-9325.

Gilbert, P. E. and R. P. Kesner (2006). "The role of the dorsal CA3 hippocampal subregion in spatial working memory and pattern separation." Behav Brain Res **169**(1): 142-149.

Gilbert, P. E., et al. (2001). "Dissociating hippocampal subregions: A double dissociation between dentate gyrus and CA1." Hippocampus **11**(6): 626-636.

Gillet, L. C., et al. (2012). "Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis." Mol Cell Proteomics **11**(6): O111 016717.

Giralt, A., et al. (2011). "Increased PKA signaling disrupts recognition memory and spatial memory: role in Huntington's disease." Hum Mol Genet **20**(21): 4232-4247.

Girouard, H., et al. (2009). "NMDA receptor activation increases free radical production through nitric oxide and NOX2." J Neurosci **29**(8): 2545-2552.

Goeldner, C., et al. (2013). "Cognitive impairment in major depression and the mGlu2 receptor as a therapeutic target." Neuropharmacology **64**: 337-346.

Gozal, E., et al. (2002). "Proteomic analysis of CA1 and CA3 regions of rat hippocampus and differential susceptibility to intermittent hypoxia." J Neurochem **83**(2): 331-345.

Gray, J. A. and B. McNaughton (1982). The Neuropsychology of anxiety.

Grentzinger, T., et al. (2012). "piRNA-mediated transgenerational inheritance of an acquired trait." Genome Research **22**(10): 1877-1888.

Grossniklaus, U., et al. (2013). "Transgenerational epigenetic inheritance: how important is it?" Nat Rev Genet **14**(3): 228-235.

Grunwald, T. and M. Kurthen (2006). "Novelty detection and encoding for declarative memory within the human hippocampus." Clin EEG Neurosci **37**(4): 309-314.

Guerrero-Bosagna, C., et al. (2012). "Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers." Reprod Toxicol **34**(4): 694-707.

Guerrero-Bosagna, C., et al. (2010). "Epigenetic Transgenerational Actions of Vinclozolin on Promoter Regions of the Sperm Epigenome." PLoS One **5**(9): e13100.

Guez-Barber, D., et al. (2012). "FACS purification of immunolabeled cell types from adult rat brain." J Neurosci Methods **203**(1): 10-18.

Gunasekar, P. G., et al. (1995). "NMDA receptor activation produces concurrent generation of nitric oxide and reactive oxygen species: implication for cell death." J Neurochem **65**(5): 2016-2021.

Guzowski, J. F., et al. (2004). "Ensemble dynamics of hippocampal regions CA3 and CA1." Neuron **44**(4): 581-584.

Guzowski, J. F., et al. (1999). "Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles." Nat Neurosci **2**(12): 1120-1124.

Gygi, S. P., et al. (1999). "Correlation between protein and mRNA abundance in yeast." Mol Cell Biol **19**(3): 1720-1730.

Hackett, J. A., et al. (2013). "Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine." Science **339**(6118): 448-452.

Hafting, T., et al. (2005). "Microstructure of a spatial map in the entorhinal cortex." Nature **436**(7052): 801-806.

Hajkova, P., et al. (2008). "Chromatin dynamics during epigenetic reprogramming in the mouse germ line." Nature **452**(7189): 877-881.

Han, S. H., et al. (2008). "Gene expression patterns in glucose-stimulated podocytes." Biochem Biophys Res Commun **370**(3): 514-518.

Harman, D. (1956). "Aging: a theory based on free radical and radiation chemistry." J Gerontol **11**(3): 298-300.

He, J., et al. (2002). "A role of Fos expression in the CA3 region of the hippocampus in spatial memory formation in rats." Neuropsychopharmacology **26**(2): 259-268.

Heckers, S. and C. Konradi (2010). "Hippocampal pathology in schizophrenia." Curr Top Behav Neurosci **4**: 529-553.

Heijmans, B. T., et al. (2008). "Persistent epigenetic differences associated with prenatal exposure to famine in humans." Proc Natl Acad Sci U S A **105**(44): 17046-17049.

Hirasawa, R., et al. (2008). "Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development." Genes Dev **22**(12): 1607-1616.

Ho, R., et al. (2013). "Stage-specific regulation of reprogramming to induced pluripotent stem cells by Wnt signaling and T cell factor proteins." Cell Rep **3**(6): 2113-2126.

Hoge, J. and R. P. Kesner (2007). "Role of CA3 and CA1 subregions of the dorsal hippocampus on temporal processing of objects." Neurobiol Learn Mem **88**(2): 225-231.

Hu, X. L., et al. (2012). "Epigenetic control on cell fate choice in neural stem cells." Protein Cell **3**(4): 278-290.

Huang, C. W., et al. (2007). "Glucose and hippocampal neuronal excitability: role of ATP-sensitive potassium channels." J Neurosci Res **85**(7): 1468-1477.

Huang, Q., et al. (2015). "SWATH enables precise label-free quantification on proteome scale." Proteomics **15**(7): 1215-1223.

Huerta, P. T., et al. (2000). "Formation of temporal memory requires NMDA receptors within CA1 pyramidal neurons." Neuron **25**(2): 473-480.

Hummon, A. B., et al. (2007). "Isolation and solubilization of proteins after TRIzol extraction of RNA and DNA from patient material following prolonged storage." BioTechniques **42**(4): 467-470, 472.

Hunsaker, M. R., et al. (2008). "Evaluating the temporal context of episodic memory: the role of CA3 and CA1." Behav Brain Res **188**(2): 310-315.

Hunter, R. G. and B. S. McEwen (2013). "Stress and anxiety across the lifespan: structural plasticity and epigenetic regulation." Epigenomics **5**(2): 177-194.

Ishihama, Y., et al. (2005). "Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein." Mol Cell Proteomics **4**(9): 1265-1272.

Johar, K., et al. (2012). "Regulation of Na(+)/K(+)-ATPase by nuclear respiratory factor 1: implication in the tight coupling of neuronal activity, energy generation, and energy consumption." J Biol Chem **287**(48): 40381-40390.

Jones, S. and J. L. Yakel (1999). "Inhibitory interneurons in hippocampus." Cell Biochem Biophys **31**(2): 207-218.

Kaczorowski, C. C. and J. F. Disterhoft (2009). "Memory deficits are associated with impaired ability to modulate neuronal excitability in middle-aged mice." Learn Mem **16**(6): 362-366.

Kanninen, K., et al. (2009). "Intrahippocampal injection of a lentiviral vector expressing Nrf2 improves spatial learning in a mouse model of Alzheimer's disease." Proc Natl Acad Sci U S A **106**(38): 16505-16510.

Karlsson, M. P. and L. M. Frank (2008). "Network dynamics underlying the formation of sparse, informative representations in the hippocampus." J Neurosci **28**(52): 14271-14281.

Kelley, A. E. and V. B. Domesick (1982). "The distribution of the projection from the hippocampal formation to the nucleus accumbens in the rat: an anterograde- and retrograde-horseradish peroxidase study." Neuroscience **7**(10): 2321-2335.

Kempadoo, K. A., et al. (2016). "Dopamine release from the locus coeruleus to the dorsal hippocampus promotes spatial learning and memory." Proc Natl Acad Sci U S A **113**(51): 14835-14840.

Kendler, K. S. (2001). "Twin studies of psychiatric illness: an update." Arch Gen Psychiatry **58**(11): 1005-1014.

Kesner, R. P., et al. (2005). "The role of CA1 in the acquisition of an object-trace-odor paired associate task." Behav Neurosci **119**(3): 781-786.

Kesner, R. P., et al. (2010). "The role of the dorsal CA1 and ventral CA1 in memory for the temporal order of a sequence of odors." Neurobiol Learn Mem **93**(1): 111-116.

Khakpai, F., et al. (2013). "Septo-hippocampo-septal loop and memory formation." Basic Clin Neurosci **4**(1): 5-23.

Khashan, A. S., et al. (2008). "Higher risk of offspring schizophrenia following antenatal maternal exposure to severe adverse life events." Arch Gen Psychiatry **65**(2): 146-152.

Kim, H. K., et al. (2009). "Intergenerational transmission of internalising and externalising behaviours across three generations: gender-specific pathways." Crim Behav Ment Health **19**(2): 125-141.

Kinney, D. K., et al. (2008). "Autism prevalence following prenatal exposure to hurricanes and tropical storms in Louisiana." J Autism Dev Disord **38**(3): 481-488.

Klengel, T., et al. (2013). "Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions." Nat Neurosci **16**(1): 33-41.

Knaus, P., et al. (1990). "Synaptoporin, a novel putative channel protein of synaptic vesicles." Neuron **5**(4): 453-462.

Knierim, J. J., et al. (2006). "Hippocampal place cells: parallel input streams, subregional processing, and implications for episodic memory." Hippocampus **16**(9): 755-764.

Kochli, D. E., et al. (2015). "The amygdala is critical for trace, delay, and contextual fear conditioning." Learn Mem **22**(2): 92-100.

Kofman, O. (2002). "The role of prenatal stress in the etiology of developmental behavioural disorders." Neurosci Biobehav Rev **26**(4): 457-470.

Kolber, B. J., et al. (2010). "Activation of metabotropic glutamate receptor 5 in the amygdala modulates pain-like behavior." J Neurosci **30**(24): 8203-8213.

Korosi, A. and T. Z. Baram (2010). "Plasticity of the stress response early in life: mechanisms and significance." Dev Psychobiol **52**(7): 661-670.

Koster, J. D., et al. (2016). "Inositol-1,4,5-trisphosphate-3-kinase-A controls morphology of hippocampal dendritic spines." Cellular Signalling **28**(1): 83-90.

Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.

Kujoth, G. C., et al. (2005). "Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging." Science **309**(5733): 481-484.

Lacy, J. W. and C. E. Stark (2013). "The neuroscience of memory: implications for the courtroom." Nat Rev Neurosci **14**(9): 649-658.

Lambert, A. J. and M. D. Brand (2009). "Reactive oxygen species production by mitochondria." Methods Mol Biol **554**: 165-181.

Lane, N., et al. (2003). "Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse." Genesis **35**(2): 88-93.

Lange, V., et al. (2008). "Selected reaction monitoring for quantitative proteomics: a tutorial." Mol Syst Biol **4**: 222.

Langston, R. F. and E. R. Wood (2010). "Associative recognition and the hippocampus: differential effects of hippocampal lesions on object-place, object-context and object-place-context memory." Hippocampus **20**(10): 1139-1153.

Larkin, M. C., et al. (2014). "Hippocampal output area CA1 broadcasts a generalized novelty signal during an object-place recognition task." Hippocampus **24**(7): 773-783.

Latham, K. E. and R. M. Schultz (2001). "Embryonic genome activation." Front Biosci **6**: D748-759.

Leak, R. K., et al. (2010). "Assaying multiple biochemical variables from the same tissue sample." J Neurosci Methods **191**(2): 234-238.

Lee, I., et al. (2005). "The role of hippocampal subregions in detecting spatial novelty." Behav Neurosci **119**(1): 145-153.

Lee, J. L., et al. (2004). "Independent cellular processes for hippocampal memory consolidation and reconsolidation." Science **304**(5672): 839-843.

Leger, M., et al. (2013). "Object recognition test in mice." Nat Protoc **8**(12): 2531-2537.

Lehmann, G., et al. (2016). "On the linkage between the ubiquitin-proteasome system and the mitochondria." Biochem Biophys Res Commun **473**(1): 80-86.

Lein, E. S., et al. (2007). "Genome-wide atlas of gene expression in the adult mouse brain." Nature **445**(7124): 168-176.

Leshem, M. and J. Schulkin (2012). "Transgenerational effects of infantile adversity and enrichment in male and female rats." Dev Psychobiol **54**(2): 169-186.

Leutgeb, J. K., et al. (2005). "Progressive transformation of hippocampal neuronal representations in "morphed" environments." Neuron **48**(2): 345-358.

Leutgeb, S., et al. (2004). "Distinct ensemble codes in hippocampal areas CA3 and CA1." Science **305**(5688): 1295-1298.

Leutgeb, S., et al. (2004). "Distinct ensemble codes in hippocampal areas CA3 and CA1." Science **305**(5688): 1295-1298.

Li, E., et al. (1993). "Role for DNA methylation in genomic imprinting." Nature **366**(6453): 362-365.

Li, J. Z., et al. (2013). "Circadian patterns of gene expression in the human brain and disruption in major depressive disorder." Proc Natl Acad Sci U S A **110**(24): 9950-9955.

Lipski, W. J. and A. A. Grace (2013). "Activation and inhibition of neurons in the hippocampal ventral subiculum by norepinephrine and locus coeruleus stimulation." Neuropsychopharmacology **38**(2): 285-292.

Lisman, J. E. and N. A. Otmakhova (2001). "Storage, recall, and novelty detection of sequences by the hippocampus: elaborating on the SOCRATIC model to account for normal and aberrant effects of dopamine." Hippocampus **11**(5): 551-568.

Lister, R., et al. (2013). "Global epigenomic reconfiguration during mammalian brain development." Science **341**(6146): 1237905.

Liu, Y., et al. (2009). "Alcohol exposure alters DNA methylation profiles in mouse embryos at early neurulation." Epigenetics **4**(7): 500-511.

Lu, B., et al. (2013). "Phosphorylation of human TFAM in mitochondria impairs DNA binding and promotes degradation by the AAA+ Lon protease." Mol Cell **49**(1): 121-132.

Lu, X., et al. (2014). "De novo transcriptome assembly for rudimentary leaves in Litchi chinesis Sonn. and identification of differentially expressed genes in response to reactive oxygen species." BMC Genomics **15**: 805.

Luger, K., et al. (1997). "Crystal structure of the nucleosome core particle at 2.8 A resolution." Nature **389**(6648): 251-260.

MacAskill, A. F., et al. (2009). "GTPase dependent recruitment of Grif-1 by Miro1 regulates mitochondrial trafficking in hippocampal neurons." Mol Cell Neurosci **40**(3): 301-312.

MacLean, B., et al. (2010). "Skyline: an open source document editor for creating and analyzing targeted proteomics experiments." Bioinformatics **26**(7): 966-968.

Macri, S., et al. (2004). "Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats." Eur J Neurosci **20**(4): 1017-1024.

Marquez, C., et al. (2013). "Peripuberty stress leads to abnormal aggression, altered amygdala and orbitofrontal reactivity and increased prefrontal MAOA gene expression." Transl Psychiatry **3**: e216.

Martig, A. K. and S. J. Mizumori (2011). "Ventral tegmental area and substantia nigra neural correlates of spatial learning." Learn Mem **18**(4): 260-271.

Maruszak, A. and C. Zekanowski (2011). "Mitochondrial dysfunction and Alzheimer's disease." Prog Neuropsychopharmacol Biol Psychiatry **35**(2): 320-330.

Matsumoto, S., et al. (2012). "Ribonucleoprotein Y-box-binding protein-1 regulates mitochondrial oxidative phosphorylation (OXPHOS) protein expression after serum stimulation through binding to OXPHOS mRNA." Biochem J **443**(2): 573-584.

McGowan, P. O., et al. (2011). "Broad epigenetic signature of maternal care in the brain of adult rats." PLoS One **6**(2): e14739.

McHugh, T. J., et al. (2007). "Dentate gyrus NMDA receptors mediate rapid pattern separation in the hippocampal network." Science **317**(5834): 94-99.

McKay, B. M., et al. (2009). "Intrinsic neuronal excitability is reversibly altered by a single experience in fear conditioning." J Neurophysiol **102**(5): 2763-2770.

McKenna, J. T. and R. P. Vertes (2001). "Collateral projections from the median raphe nucleus to the medial septum and hippocampus." Brain Res Bull **54**(6): 619-630.

Mendez, M., et al. (2015). "c-Fos expression correlates with performance on novel object and novel place recognition tests." Brain Res Bull **117**: 16-23.

Menzel, C. R. (1999). "Unprompted recall and reporting of hidden objects by a chimpanzee (Pan troglodytes) after extended delays." J Comp Psychol **113**(4): 426-434.

Mery, F. (2013). "Natural variation in learning and memory." Curr Opin Neurobiol **23**(1): 52-56.

Millan, M. J., et al. (2012). "Cognitive dysfunction in psychiatric disorders: characteristics, causes and the quest for improved therapy." Nat Rev Drug Discov **11**(2): 141-168.

Miller, C. A. and J. D. Sweatt (2007). "Covalent modification of DNA regulates memory formation." Neuron **53**(6): 857-869.

Molfese, D. L. (2011). "Advancing Neuroscience Through Epigenetics: Molecular Mechanisms of Learning and Memory." Developmental Neuropsychology **36**(7): 810-827.

Mongillo, G., et al. (2008). "Synaptic theory of working memory." Science **319**(5869): 1543-1546.

Monopoli, M. P., et al. (2011). "Temporal proteomic profile of memory consolidation in the rat hippocampal dentate gyrus." Proteomics **11**(21): 4189-4201.

Moreira, P. I., et al. (2010). "Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology." Biochim Biophys Acta **1802**(1): 2-10.

Morgan, C. P. and T. L. Bale (2011). "Early Prenatal Stress Epigenetically Programs Dysmasculinization in Second-Generation Offspring via the Paternal Lineage." The Journal of Neuroscience **31**(33): 11748-11755.

Morris, R. G., et al. (2006). "Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval." Neuron **50**(3): 479-489.

Moser, E., et al. (1993). "Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions." J Neurosci **13**(9): 3916-3925.

Moser, E. I., et al. (2008). "Place cells, grid cells, and the brain's spatial representation system." Annu Rev Neurosci **31**: 69-89.

Moser, M. B., et al. (1995). "Spatial learning with a minislab in the dorsal hippocampus." Proc Natl Acad Sci U S A **92**(21): 9697-9701.

Moyer, J. R., Jr., et al. (1996). "Trace eyeblink conditioning increases CA1 excitability in a transient and learning-specific manner." J Neurosci **16**(17): 5536-5546.

Mueller, B. R. and T. L. Bale (2007). "Early prenatal stress impact on coping strategies and learning performance is sex dependent." Physiol Behav **91**(1): 55-65.

Mueller, B. R. and T. L. Bale (2008). "Sex-specific programming of offspring emotionality after stress early in pregnancy." J Neurosci **28**(36): 9055-9065.

Nader, K. and O. Hardt (2009). "A single standard for memory: the case for reconsolidation." Nat Rev Neurosci **10**(3): 224-234.

Nakazawa, K., et al. (2002). "Requirement for hippocampal CA3 NMDA receptors in associative memory recall." Science **297**(5579): 211-218.

Nandi, D., et al. (2006). "The ubiquitin-proteasome system." J Biosci **31**(1): 137-155.

Navarro, A. and A. Boveris (2010). "Brain mitochondrial dysfunction in aging, neurodegeneration, and Parkinson's disease." Front Aging Neurosci **2**.

Navarro, A., et al. (2008). "Hippocampal mitochondrial dysfunction in rat aging." Am J Physiol Regul Integr Comp Physiol **294**(2): R501-509.

Nestler, E. J. (2013). "Epigenetic mechanisms of drug addiction." Neuropharmacology.

Newrzella, D., et al. (2007). "The functional genome of CA1 and CA3 neurons under native conditions and in response to ischemia." BMC Genomics **8**: 370.

Nithianantharajah, J. and A. J. Hannan (2006). "Enriched environments, experience-dependent plasticity and disorders of the nervous system." Nat Rev Neurosci **7**(9): 697-709.

Nitz, D. and B. McNaughton (2004). "Differential modulation of CA1 and dentate gyrus interneurons during exploration of novel environments." J Neurophysiol **91**(2): 863-872.

O'Keefe, J. (1976). "Place units in the hippocampus of the freely moving rat." Exp Neurol **51**(1): 78-109.

Ogawa, K. and T. Inui (2007). "Lateralization of the posterior parietal cortex for internal monitoring of self- versus externally generated movements." Journal of cognitive neuroscience **19**(11): 1827-1835.

Oh, M. M., et al. (2010). "Learning and aging related changes in intrinsic neuronal excitability." Front Aging Neurosci **2**: 2.

Oh, M. M., et al. (2016). "Intrinsic Hippocampal Excitability Changes of Opposite Signs and Different Origins in CA1 and CA3 Pyramidal Neurons Underlie Aging-Related Cognitive Deficits." Front Syst Neurosci **10**: 52.

Oliveira-da-Silva, A., et al. (2009). "Increased apoptosis and reduced neuronal and glial densities in the hippocampus due to nicotine and ethanol exposure in adolescent mice." Int J Dev Neurosci **27**(6): 539-548.

Oswald, J., et al. (2000). "Active demethylation of the paternal genome in the mouse zygote." Current Biology **10**(8): 475-478.

Ota, Y., et al. (2013). "The role of astrocytes in the regulation of synaptic plasticity and memory formation." Neural Plast **2013**: 185463.

Park, A. J., et al. (2014). "A presynaptic role for PKA in synaptic tagging and memory." Neurobiol Learn Mem **114**: 101-112.

Parra, P., et al. (1998). "How many subtypes of inhibitory cells in the hippocampus?" Neuron **20**(5): 983-993.

Payne, B. A. and P. F. Chinnery (2015). "Mitochondrial dysfunction in aging: Much progress but many unresolved questions." Biochim Biophys Acta **1847**(11): 1347-1353.

Peleg-Raibstein, D., et al. (2012). "Maternal high-fat diet in mice programs emotional behavior in adulthood." Behav Brain Res **233**(2): 398-404.

Prasad, A. and C. Prasad (1996). "Short-term consumption of a diet rich in fat decreases anxiety response in adult male rats." Physiol Behav **60**(3): 1039-1042.

Przybyslawski, J. and S. J. Sara (1997). "Reconsolidation of memory after its reactivation." Behav Brain Res **84**(1-2): 241-246.

Radford, E. J., et al. (2012). "An unbiased assessment of the role of imprinted genes in an intergenerational model of developmental programming." PLoS Genet **8**(4): e1002605.

Rajji, T., et al. (2006). "The role of CA3 hippocampal NMDA receptors in paired associate learning." Journal of Neuroscience **26**(3): 908-915.

Rajji, T., et al. (2006). "The role of CA3 hippocampal NMDA receptors in paired associate learning." J Neurosci **26**(3): 908-915.

Rao-Ruiz, P., et al. (2015). "Time-dependent changes in the mouse hippocampal synaptic membrane proteome after contextual fear conditioning." Hippocampus **25**(11): 1250-1261.

Rassoulzadegan, M., et al. (2006). "RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse." Nature **441**(7092): 469-474.

Ravelli, A. C., et al. (1998). "Glucose tolerance in adults after prenatal exposure to famine." Lancet **351**(9097): 173-177.

Ravelli, A. C., et al. (1999). "Obesity at the age of 50 y in men and women exposed to famine prenatally." Am J Clin Nutr **70**(5): 811-816.

Razy-Krajka, F., et al. (2014). "Collier/OLF/EBF-dependent transcriptional dynamics control pharyngeal muscle specification from primed cardiopharyngeal progenitors." Dev Cell **29**(3): 263-276.

Rechavi, O., et al. (2011). "Transgenerational inheritance of an acquired small RNA-based antiviral response in C. elegans." Cell **147**(6): 1248-1256.

Reiter, L., et al. (2011). "mProphet: automated data processing and statistical validation for large-scale SRM experiments." Nature Methods **8**(5): 430-435.

Roberts, A. L., et al. (2012). "Posttraumatic stress disorder across two generations: concordance and mechanisms in a population-based sample." Biol Psychiatry **72**(6): 505-511.

Robinson, V. (2005). "Finding alternatives: an overview of the 3Rs and the use of animals in research." School Sci Rev **87**: 1-4.

Rodgers, A. B., et al. (2013). "Paternal Stress Exposure Alters Sperm MicroRNA Content and Reprograms Offspring HPA Stress Axis Regulation." J Neurosci **33**(21): 9003-9012.

Roseboom, T. J., et al. (2000). "Plasma lipid profiles in adults after prenatal exposure to the Dutch famine." Am J Clin Nutr **72**(5): 1101-1106.

Ross, P. L., et al. (2004). "Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents." Mol Cell Proteomics **3**(12): 1154-1169.

Roth, T. L., et al. (2009). "Lasting Epigenetic Influence of Early-Life Adversity on the BDNF Gene." Biol Psychiatry **65**(9): 760-769.

Saab, B. J., et al. (2011). "Statistical and theoretical considerations for the platform re-location water maze." J Neurosci Methods **198**(1): 44-52.

Sakurai, Y. (1996). "Hippocampal and neocortical cell assemblies encode memory processes for different types of stimuli in the rat." J Neurosci **16**(8): 2809-2819.

Samara, A., et al. (2011). "Proteomics reveal rat hippocampal lateral asymmetry." Hippocampus **21**(1): 108-119.

Sandhu, S. K. and G. Kaur (2003). "Mitochondrial electron transport chain complexes in aging rat brain and lymphocytes." Biogerontology **4**(1): 19-29.

Santos, F., et al. (2002). "Dynamic reprogramming of DNA methylation in the early mouse embryo." Dev Biol **241**(1): 172-182.

Santos, F., et al. (2005). "Dynamic chromatin modifications characterise the first cell cycle in mouse embryos." Dev Biol **280**(1): 225-236.

Santos, S. F., et al. (2010). "Network excitability dysfunction in Alzheimer's disease: insights from in vitro and in vivo models." Rev Neurosci **21**(3): 153-171.

Sarel, A., et al. (2017). "Vectorial representation of spatial goals in the hippocampus of bats." Science **355**(6321): 176-180.

Sasaki, A., et al. (2013). "Perinatal high fat diet alters glucocorticoid signaling and anxiety behavior in adulthood." Neuroscience **240**: 1-12.

Sasaki, H. and Y. Matsui (2008). "Epigenetic events in mammalian germ-cell development: reprogramming and beyond." Nat Rev Genet **9**(2): 129-140.

Scarpulla, R. C. (1997). "Nuclear control of respiratory chain expression in mammalian cells." J Bioenerg Biomembr **29**(2): 109-119.

Scholz, B., et al. (2008). "Striatal proteomic analysis suggests that first L-dopa dose equates to chronic exposure." PLoS One **3**(2): e1589.

Schwartz, B. L. and S. Evans (2001). "Episodic memory in primates." Am J Primatol **55**(2): 71-85.

Schwarz, T. L. (2013). "Mitochondrial trafficking in neurons." Cold Spring Harb Perspect Biol **5**(6).

Scorcioni, R., et al. (2004). "Quantitative morphometry of hippocampal pyramidal cells: differences between anatomical classes and reconstructing laboratories." J Comp Neurol **473**(2): 177-193.

Scoville, W. B. and B. Milner (1957). "Loss of recent memory after bilateral hippocampal lesions." J Neurol Neurosurg Psychiatry **20**(1): 11-21.

Seki, Y., et al. (2005). "Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice." Dev Biol **278**(2): 440-458.

Selevsek, N., et al. (2015). "Reproducible and consistent quantification of the Saccharomyces cerevisiae proteome by SWATH-mass spectrometry." Mol Cell Proteomics **14**(3): 739-749.

Sevigny, J., et al. (2016). "The antibody aducanumab reduces Abeta plaques in Alzheimer's disease." Nature **537**(7618): 50-56.

Shah, S., et al. (2016). "In Situ Transcription Profiling of Single Cells Reveals Spatial Organization of Cells in the Mouse Hippocampus." Neuron **92**(2): 342-357.

Skinner, M. K. (2008). "What is an epigenetic transgenerational phenotype? F3 or F2." Reprod Toxicol **25**(1): 2-6.

Skinner, M. K., et al. (2008). "Transgenerational Epigenetic Programming of the Brain Transcriptome and Anxiety Behavior." PLoS One **3**(11): e3745.

Skinner, M. K., et al. (2013). "Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line." PLoS One **8**(7): e66318.

Smith, B. R. B. D. T. (2008). "Fitness consequences of personality: a meta-analysis." Behavioral Ecology.

Smith, C. A., et al. (2005). "Adolescent maltreatment and its impact on young adult antisocial behavior." Child Abuse Negl **29**(10): 1099-1119.

Solaini, G., et al. (2010). "Hypoxia and mitochondrial oxidative metabolism." Biochim Biophys Acta **1797**(6-7): 1171-1177.

Soubry, A., et al. (2013). "Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort." BMC Med **11**: 29.

Spear, L. P. (2009). "Heightened stress responsivity and emotional reactivity during pubertal maturation: Implications for psychopathology." Dev Psychopathol **21**(1): 87-97.

Squire, L. R. (2006). "Lost forever or temporarily misplaced? The long debate about the nature of memory impairment." Learn Mem **13**(5): 522-529.

Squire, L. R. and P. Alvarez (1995). "Retrograde amnesia and memory consolidation: a neurobiological perspective." Curr Opin Neurobiol **5**(2): 169-177.

Stein, A. D., et al. (2009). "Maternal exposure to the Dutch famine before conception and during pregnancy: quality of life and depressive symptoms in adult offspring." Epidemiology **20**(6): 909-915.

Stokes, W. S., et al. (2012). "Recent progress and future directions for reduction, refinement, and replacement of animal use in veterinary vaccine potency and safety testing: a report from the 2010 NICEATM-ICCVAM International Vaccine Workshop." Dev Biol (Basel) **134**: 9-21.

Stouder, C. and A. Paoloni-Giacobino (2010). "Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm." Reproduction **139**(2): 373-379.

Strange, B. A., et al. (2014). "Functional organization of the hippocampal longitudinal axis." Nat Rev Neurosci **15**(10): 655-669.

Sullivan, P. F., et al. (2000). "Genetic epidemiology of major depression: review and meta-analysis." Am J Psychiatry **157**(10): 1552-1562.

Susser, E., et al. (1996). "Schizophrenia after prenatal famine. Further evidence." Arch Gen Psychiatry **53**(1): 25-31.

Taube, J. S. (1998). "Head direction cells and the neurophysiological basis for a sense of direction." Prog Neurobiol **55**(3): 225-256.

Thompson, C. L., et al. (2008). "Genomic anatomy of the hippocampus." Neuron **60**(6): 1010-1021.

Tobe, E. H. (2013). "Mitochondrial dysfunction, oxidative stress, and major depressive disorder." Neuropsychiatr Dis Treat **9**: 567-573.

Tobi, E. W., et al. (2009). "DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific." Hum Mol Genet **18**(21): 4046-4053.

Tobi, E. W., et al. (2012). "Prenatal famine and genetic variation are independently and additively associated with DNA methylation at regulatory loci within IGF2/H19." PLoS One **7**(5): e37933.

Tolosa, J. M., et al. (2007). "Column-based method to simultaneously extract DNA, RNA, and proteins from the same sample." BioTechniques **43**(6): 799-804.

Tost, J. (2009). "DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker." Methods Mol Biol **507**: 3-20.

Trifunovic, A., et al. (2004). "Premature ageing in mice expressing defective mitochondrial DNA polymerase." Nature **429**(6990): 417-423.

Tsankova, N. M., et al. (2006). "Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action." Nat Neurosci **9**(4): 519-525.

Tweedie-Cullen, R. Y., et al. (2012). "Identification of combinatorial patterns of post-translational modifications on individual histones in the mouse brain." PLoS One **7**(5): e36980.

Tweedie-Cullen, R. Y., et al. (2009). "Comprehensive Mapping of Post-Translational Modifications on Synaptic, Nuclear, and Histone Proteins in the Adult Mouse Brain." Journal of Proteome Research **8**(11): 4966-4982.

Uchida, S., et al. (2011). "Epigenetic status of Gdnf in the ventral striatum determines susceptibility and adaptation to daily stressful events." Neuron **69**(2): 359-372.

Van der Zee, E. A., et al. (2005). "TGFalpha and AVP in the mouse suprachiasmatic nucleus: anatomical relationship and daily profiles." Brain Res **1054**(2): 159-166.

van Groen, T., et al. (2003). "The entorhinal cortex of the mouse: organization of the projection to the hippocampal formation." Hippocampus **13**(1): 133-149.

van Os, J. and J. P. Selten (1998). "Prenatal exposure to maternal stress and subsequent schizophrenia. The May 1940 invasion of The Netherlands." Br J Psychiatry **172**: 324-326.

VanElzakker, M., et al. (2008). "Environmental novelty is associated with a selective increase in Fos expression in the output elements of the hippocampal formation and the perirhinal cortex." Learn Mem **15**(12): 899-908.

Vanelzakker, M. B., et al. (2011). "Influence of Pre-Training Predator Stress on the Expression of c-fos mRNA in the Hippocampus, Amygdala, and Striatum Following Long-Term Spatial Memory Retrieval." Front Behav Neurosci **5**: 30.

Vann, S. D. and M. M. Albasser (2011). "Hippocampus and neocortex: recognition and spatial memory." Curr Opin Neurobiol **21**(3): 440-445.

Vassoler, F. M., et al. (2013). "Epigenetic inheritance of a cocaine-resistance phenotype." Nat Neurosci **16**(1): 42-47.

Veenit, V., et al. (2013). "Increased corticosterone in peripubertal rats leads to long-lasting alterations in social exploration and aggression." Front Behav Neurosci **7**: 26.

Vogel-Ciernia, A. and M. A. Wood (2014). "Examining object location and object recognition memory in mice." Curr Protoc Neurosci **69**: 8 31 31-17.

WAIS (2006). WAIS, 2006. Animal Welfare act. Congressional statement of policy. United States of America.

Wan, M., et al. (2013). "Inducible mouse models illuminate parameters influencing epigenetic inheritance." Development **140**(4): 843-852.

Weaver, I. C., et al. (2004). "Epigenetic programming by maternal behavior." Nat Neurosci **7**(8): 847-854.

Weiss, I. C., et al. (2011). "Inheritable effect of unpredictable maternal separation on behavioral responses in mice." Front Behav Neurosci **5**: doi: 10.3389/fnbeh.2011.00003.

Weissman, M. M., et al. (2005). "Families at high and low risk for depression: a 3-generation study." Arch Gen Psychiatry **62**(1): 29-36.

Wilson, M. A. and B. L. McNaughton (1993). "Dynamics of the hippocampal ensemble code for space." Science **261**(5124): 1055-1058.

Winklhofer, K. F. and C. Haass (2010). "Mitochondrial dysfunction in Parkinson's disease." Biochim Biophys Acta **1802**(1): 29-44.

Winocur, G. and M. Moscovitch (2011). "Memory transformation and systems consolidation." J Int Neuropsychol Soc **17**(5): 766-780.

Winograd, M. and H. Viola (2004). "Detection of novelty, but not memory of spatial habituation, is associated with an increase in phosphorylated cAMP response element-binding protein levels in the hippocampus." Hippocampus **14**(1): 117-123.

Wisniewski, J. R., et al. (2009). "Universal sample preparation method for proteome analysis." Nature Methods **6**(5): 359-362.

Witter, M. P., et al. (1989). "Functional organization of the extrinsic and intrinsic circuitry of the parahippocampal region." Prog Neurobiol **33**(3): 161-253.

Wolstenholme, J. T., et al. (2012). "Gestational Exposure to Bisphenol A Produces Transgenerational Changes in Behaviors and Gene Expression." Endocrinology **153**(8): 3828-3838.

Yang, G., et al. (2009). "Stably maintained dendritic spines are associated with lifelong memories." Nature **462**(7275): 920-924.

Yang, S. J., et al. (2006). "Activity-dependent transcriptional regulation of nuclear respiratory factor-1 in cultured rat visual cortical neurons." Neuroscience **141**(3): 1181-1192.

Yonelinas, A. P., et al. (2002). "Effects of extensive temporal lobe damage or mild hypoxia on recollection and familiarity." Nat Neurosci **5**(11): 1236-1241.

Zellner, M., et al. (2005). "Quantitative validation of different protein precipitation methods in proteome analysis of blood platelets." Electrophoresis **26**(12): 2481-2489.

Zhang, B., et al. (2005). "WebGestalt: an integrated system for exploring gene sets in various biological contexts." Nucleic Acids Res **33**(Web Server issue): W741-748.

Zhang, L., et al. (2006). "Stress-induced change of mitochondria membrane potential regulated by genomic and non-genomic GR signaling: a possible mechanism for hippocampus atrophy in PTSD." Med Hypotheses **66**(6): 1205-1208.

Zhu, X. H., et al. (2012). "Quantitative imaging of energy expenditure in human brain." Neuroimage **60**(4): 2107-2117.

Zmigrod, S. and B. Hommel (2013). "Feature integration across multimodal perception and action: a review." Multisens Res **26**(1-2): 143-157.

Zovkic, I. B., et al. (2013). "Epigenetic regulation of memory formation and maintenance." Learn Mem **20**(2): 61-74.

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